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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT und r 37 CFR 1.53(c).

Expr ss Mail Label No.

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

VACCINATION VECTORS DERIVED FROM LYMPHOTROPIC HUMAN HERPES VIRUSES 6 AND 7

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☐ Application Data Sheet. See 37 CFR 1.76
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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted

SIGNATURE _____

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Date Aug. 4, 2003

REGISTRATION NO. *(if appropriate)* 40,669

Docket Number: 25626

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This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

MAIL STOP PROVISIONAL PATENT APPLICATION
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Niza FRENKEL

Serial No. NOT YET ASSIGNED

Filed: August 4, 2003

For: **VACCINATION VECTORS DERIVED FROM LYMPHOTROPIC HUMAN HERPES VIRUSES 6 AND 7**

TRANSMITTAL LETTER

Commissioner for Patents
Mail Stop Provisional Patent Application
P.O. Box 1450
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Sir:

Submitted herewith for filing in the U.S. Patent and Trademark Office is the following **PROVISIONAL APPLICATION:**

(1) Transmittal Letter
(2) Cover sheet for filing **Provisional Application**
(3) 65 page Provisional Application consisting of:
45 pages Textual Specification,
2 pages of Claims,
18 sheets of Drawings;
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The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,
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VACCINATION VECTORS DERIVED FROM LYMPHOTROPIC HUMAN HERPES VIRUSES 6 AND 7

FIELD OF THE INVENTION

The present invention is generally in the field of viral vectors as vaccination vectors.

BACKGROUND OF THE INVENTION

PRIOR ART

The following are references considered to be relevant for the subsequent description.

Ablashi D, A. H., Balachandran, N., Josephs, S.F., Hung, C.L., Krueger G.R., Kramarsky, B., Salahuddin S.Z. and Gallo R.C. (1991). Genomic polymorphism, growth properties, and immunologic variations in human herpesvirus-6 isolates. Virology 184:545-552.

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Frenkel, N., E.C. Schirmer, Wyatt L,. S. Katsafanas G, Roffman, E., Danovich R.M. and June, C.H. (1990) Isolation of a new herpesvirus from human CD4+ T cells. Proc. Natl. Acad. Sci. USA. 87:748-752.

Frenkel, N., Katsafanas, G. C., Wyatt, L. S., Yoshikawa, T., and Asano, Y. (1994). Bone marrow transplant recipients harbor the B variant of human herpesvirus 6. Bone Marrow Transplant 14:839-43.

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Romi H., Singer O., Rapaport D., and Frenkel N. (1999), Tamplicon-7, a novel T-lymphotropic vector derived from human herpervirus 7. *J. Virol.* **73**:7001-7.

Schirmer E.C., Wyatt L.S., Yamanishi K., Rodriguez W.J., and Frenkel N. (1991) Differentiation between two distinct classes of viruses now classified as human herpes virus 6. *Proc. Natl. Acad. Sci., USA*, **88**:199-208.

US 5,230,997 Methods of detecting the presence of human herpesvirus-7 infection.

US 6,503,752, Lymphotropic agents and vectors.

US 6,544,780, Adenovirus vector with multiple expression cassettes.

WO 99/07869, Live recombinant vaccine comprising inefficiently or non-replicating virus.

Wyatt L., and Frenkel N. (1992) Human herpes virus 7 is a constitutive inhabitant of adult human saliva. *J. Virol.* **66**:3206-3209.

Yamanishi, K. (1992). Human herpesvirus 6. Microbiol Immunol 36:551-61.

Human herpes virus-6 (HHV-6) was first isolated from peripheral blood mononucleur cells (PBMC) of patients with lympho-proliforative disorders as

well as from patients suffering from acquired immune deficiency syndrome (AIDS).

Two types of HHV-6 strains are recognized today and designated as variant A and variant B. They are closely related variants with DNA sequence homology ranging from 75 to 97%, depending on the gene(s). They differ regarding their growth properties, restriction enzyme patterns and antigenicity and they are also distinct epidemiologically (Pellet, 2002, Schirmer et al. 1991, Ablashi et al., 1991). Only the HHV-6B variant appears to be be associated with human diseases. It infects the majority of children during the first 2 years of life. The virus causes roseola infantum or Exanthem Subitum (ES), usually a mild disease, characterized by several days of spiky fever and skin rash (Yamanishi et al., 1988). In some ES patients, the disease can extend to the central nervous system (CNS), up to fatal fulminate hepatitis. Furthermore, reactivation of HHV-6B from latency could play a role in some post-transplant complications, especially in patients with impaired immune capabilities, including AIDS patients and patients receiving preparatory immunosuppressive therapy in bone marrow transplantation (BMT) (Rapaport et al., 2002). The HHV-6 reactivation can cause late engraftment, up to lethal encephalitis.

In contrast to disease association of HHV-6B, symptomatic HHV-6A infections in children are rather rare and the virus is not known to be associated with children's diseases or in reactivation from latency in transplanted patients (Frenkel et al., 1994, Schirmer et al., 1991).

Human herpes virus-7 (HHV-7) is a DNA virus first isolated in the laboratory of the inventor of the present invention from activated T cells expressing the CD4 antigen (see US 5,230,997, Romi *et al.* 1999, Frenkel *et al.*, 1990). Cells expressing this antigen on their membrane will hereinafter be referred to as *"$CD4^+$ cells"*. The virus uses CD4 as an entry receptor.

HHV-7 was found to be distinct, both molecularly and antigenically, from all previously identified herpes viruses. HHV-7 replicates well in lymphocytes and particularly in T cells including $CD4^+$ T cells and possibly other cells carrying the CD4 marker.

HHV-7 can persistently infect salivary glands, and it is continuously secreted into the saliva of more than 95% of humans (Wyatt and Frenkel, 1992). Although the virus infects the majority of children in early childhood, no known disease is associated with the virus. Latent virus genomes can be identified in many healthy individuals, and the virus can be activated from latency in vitro, by exposing the T cells to activation conditions (Katsafanas et al., 1996). No HHV – 7 reactivation has been reported in bone marrow transplantation (Rappaport et al., 2002).

The HHV-6A, HHV-6B and HHV-7 genomes are linear, double-stranded DNA molecules of 162-170Kb. The genomes are composed of a 143Kb segment of unique (U) sequences, bracketed by direct repeats DR_L (left) and DR_R (right), (Pellet et al. 2002). The viral genomes have similar arrangement of genes across the genomes (Pellet 2002.). HHV 6A, 6B and 7 each have a single DNA replication origin (oriLyt) (Dewhurst 1993; Romi et al., 1999; Pellet 2002) which replicates in the nucleus by the rolling circle mechanism, as shown by Frenkel's group (Romi et al, 1999). The DR sequences are bound by the pac-1 and pac-2 herpesvirus conserved packaging signals (Frenkel and Roffman, 1996). The genome circularizes prior to the rolling circle replication, which leads to the formation of a complete pac-1-pac-2 cleavage/packaging signal. The consequent rolling circle replication generates large concatameric molecules, with pac-1-pac-2 signals bounding the repeats. The HSV amplicons, amplicon-6 and Tamplicon-7 vectors derived from HSV-1, HHV-6 and HHV-7, respectively, were previously described (US 6,503,752). The constructed vectors contain a viral DNA replication origin, cleavage and packaging signals and the transgene(s). In the presence of helper virus functions the amplicon plasmid is replicated by the rolling circle mechanism and generates huge concatameric genomes which can be cleaved between the pac-1 and pac-2 signals. The most efficient cleavage occurs when the DNA molecules reach approximately full length 135-150Kb genomes, made of identical amplicon repeats. The packaged conamplicons are replication defective, but can enter into new cells and express their transgenes at high efficiency, due to sequence reiteration.

During packaging the concatamers are cleaved and packaged at 29-35bp from pac-2, and 41-46bp from pac-1 signals (Frenkel and Roffman, 1996; Romi et al., 1999). This process is most efficient for full-length DNA genoes (i.e. 135-150K0). The capsids acquire the tegument layer in intranuclear tegusome structures, after which the particles appear to be released into the cytoplasm via fusion with the nuclear membrane. Envelopment occurs by budding into cytoplasmic vacuoles, which then fuse with the cell membrane to release mature particles (Roffman et al., 1990). The pac-1 and pac-2 signals are necessary for the entry of the packaged DNA into the cytoplasm and for further exit out of the cells and into the medium. The rolling circle mechanism and consequent cleavage and packaging processes are utilized in the production of the defective virus amplicon vectors.

Vaccinations have traditionally included injecting into the body an attenuated or killed form of a bacterium or virus, or injection of denatured proteins. While efficient in many cases, this form of vaccination is not effective in other cases, such as integral membrane proteins, HIV-related proteins, etc. Furthermore, such vaccines raise concerns regarding the ability of a live virus to establish latency, to reactivate, and to recombine with virulent wild type viruses, in addition to concerns regarding the oncogenic potential of some viral genes.

Another approach is DNA vaccination, whereby the DNA that encodes the desired protein to which immunity is sought is injected into the body, usually as part of a plasmid. This vaccination mode is often inefficient and costly.

Recently, another vaccination approach – genetic vaccination, has been formulated. Here, a known viruse is mutated in order to render it benign, and the virus then serves as a vector for introducing a cargo gene of interest into the host's cells. The gene is translated and expressed by the cells, and the protein product may induce an immune response in the host. Viruses are more efficient as vaccination vehicles since they enter their host cells with efficiency, and may also replicate in the cells thereby increasing the level of expression of their cargo gene.

Genetic vaccination has been described using the Vaccinia virus and mutants thereof, mainly the modified Vaccinia virus Ankara (MVA, WO9907869) and the Adenovirus (US6544780). Unfortunately, Vaccinia has been shown to cause complications in individuals who were previously vaccinated against smallpox, and immune memory in individuals who have previously received Vaccinia virus may prevent recognition of any foreign gene insert (McDermott *et al.*, 1989).

Hanke et al. (2003) describe a human immunodeficiency virus (HIV) vaccine that consists of Semliki Forest virus (SFV), and a cargo gene encoding HIVA, which is an immunogen derived from HIV-1 clade A. In the mouse, the SFV.HIVA vaccine induced T cell-mediated immune responses and induced T cell memory that lasted for at least 6 months. However, SFV.HIVA is even less immunogenic than modified Vaccinia virus Ankara carrying HIVA (MVA.HIVA).

GLOSSARY

Vector refers to a DNA molecule capable of carrying a foreign nucleic acid sequence of interest (see below). This includes the DNA vector *per se*, as well as the vector that is packaged in a virion particle. The vector includes an origin of replication, a promoter sequence which allows expression in a host and a cleavage and packaging signal.

Lymphotropic Vector refers to a vector that is specifically capable of being expressed in lymphatic cells. This includes the DNA vector *per se*, as well as the vector that is packaged in a virion particle, in which case the targeting of the vector will be more efficient. Lymphatic cells include T cells, B cells, monocytes, macrophages, NK cytotoxic T cells (CTL) and dendritic cells. When the lymphotropic vector is amplicon-6, it is also capable of being targeted to and expressed in $CD4^+$- cells of other, non-lymphatic origin.

Transgene or ***foreign nucleic acid*** refers to a nucleic acid sequence encoding a protein of interest, that is inserted into the vector of the invention. At times, the transgene will be referred to simply as a "***gene***". By "*nucleic acid sequence encoding a protein of interest*" is meant a sequence of a known gene of interest, including both the genomic sequence and the mRNA sequence, as well as sequences controlling the expression level of the mRNA or protein. This definition further comprises any modification of said sequence, including deleti, mutations, introduction of cellular transport-specific signals as are known in the art (e.g. a membrane-targeting signal, or signal peptide; ER or Golgi targeting signals, etc.), or fragments of at least 20 base pairs (bp) thereof. Also included are sequences complementary to said nucleic acid sequences, i.e. antisense sequences.

Eliciting an immune response or ***inducing an immune response*** refers to activating either the humoral arm or the cellular arm of the immune system, or both. At times, this will also be referred to as "***vaccination.*** Activation of the immune system may be assessed by any method known in the art, including production of antibodies and neutralizing antibodies; production or secretion of specific proteins such as interleukins, interferon, tumor necrosis factor (TNF) and any other indicators known in the art; and eliciting chemokines and cytokines known to attract lymphocytes and cytotoxic T cells to the site of infection.

Defective genome or ***replication-defective genome*** or ***defective virus*** all refer to a virus particle that is incapable of autonomous replication in a host cell. In particular, such a definition comprises the amplicon-6 and Tamplicon-7 vectors. Viral particles that have a defective genome will need a helper virus in order to replicate in a host cell.

Membrane associated refers to protein products that either have a transmembrane domain, or are capable of being modified in the cell such that they will be associated with the cell membrane. At times, these proteins will

also be referred to as cell-surface associated proteins or proteins underlying the cell membrane. Among the known modifications, typical, but not exclusive examples include: acylation, amidation, GPI anchor formation, covalent attachment of a lipid or lipid derivative, myristoylation, pegylation, prenylation, palmitoylation, methylation, or any similar process.

"***Nucleic acid molecule***" or "***nucleic acid***" denotes a single-stranded or double-stranded polymer composed of DNA nucleotides, RNA nucleotides or a combination of both types and may include natural nucleotides, chemically modified nucleotides and synthetic nucleotides. This includes also oligomers and PCR primers.

"***Amino acid sequence***" a sequence composed of any one of the 20 naturally appearing amino acids, amino acids which have been *chemically modified* (see below), or synthetic amino acids.

"***Antibody***" - refers to antibodies of any of the classes IgG, IgM, IgD, IgA, and IgE antibody. The definition includes polyclonal antibodies or monoclonal antibodies. This term refers to whole antibodies or fragments of the antibodies comprising the antigen-binding domain of the anti-variant product antibodies, e.g. scFv, Fab, $F(ab')_2$, other antibodies without the Fc portion, single chain antibodies, bispecific antibodies, diabodies, other fragments consisting of essentially only the variable, antigen-binding domain of the antibody, etc., which substantially retain the antigen-binding characteristics of the whole antibody from which they were derived. This definition also includes recombinant or synthetic antibodies and antibodies carrying toxic genes.

"Treating a disease" - refers to administering a therapeutic substance effective to prevent or ameliorate symptoms associated with a disease, to lessen the severity or cure the disease, or to prevent the disease from occurring. Treatment may also refer to slowing down the progression of the disease or the

deterioration of the symptoms associated therewith, to enhancing the onset of the remission period, to slowing down the irreversible damage caused in the progressive chronic stage of the disease, to delaying the onset of said progressive stage, to improving survival rate or more rapid recovery, or a combination of two or more of the above.

The treatment regimen will depend on the type of disease to be treated and may be determined by various considerations known to those skilled in the art of medicine, e.g. the physicians.

"***Effective amount***" for purposes herein is determined by such considerations as may be known in the art. The amount must be effective to achieve the desired therapeutic effect as described above, i.e. eliciting an appropriate immune response. The amount depends, among other things, on the type and severity of the disease to be treated and the treatment regime. The effective amount is typically determined in appropriately designed clinical trials (dose range studies) and the person versed in the art will know how to properly conduct such trials in order to determine the effective amount. As generally known, an effective amount depends on a variety of factors including the efficiency of expression of the desired protein, the efficiency of induction of an immune response against said protein, a variety of pharmacological parameters such as half life in the body, undesired side effects, if any, factors such as age and gender of the treated individual, etc.

"***Pharmaceutically acceptable carrier***" means any inert, non-toxic material, which does not react with the vectors of the invention. Thus, the carrier can be any of those conventionally used and is limited only by chemico-physical considerations, such as solubility and lack of reactivity with the compound, and by the route of administration. Examples of pharmaceutically acceptable carriers are detailed later on.

SUMMARY OF THE INVENTION

The present invention concerns the use of amplicon-6 and Tamplicon–7 as vaccination vectors for efficient expression of selected genes in human lymphocytes. The main characteristics of the vectors of the invention are:

(i) The vectors contain defective genomes of a total size close to 150kb made of multiple reiterations of amplicon units with the capacity to introduce long foreign DNA sequences. An amplicon may contain 10 reiterations of 15 kb repeat units. One could place several genes inside this unit, such as the gD and gDsec genes (see below), as well as Interferon. Gene expression is efficient due to sequence reiterations.

(ii) The host range of the HHV-6 and HHV-7 vectors includes T cells, B cells, monocytes as well as dendritic cells. This is advantageous for vaccination inasmuch as lymphocytes express high levels of MHC class I molecules and induce strong immune response(s); the dendritic cells are efficient antigen presenting cells (APC).

(iii) HHV-6A and HHV-7 are prevalent viruses which cause no known disease. The majority of children show HHV-6 seroconversion before the age of 2 years. More than 80% of healthy adults are persistently infected with HHV-7 and secrete the virus in their saliva.

(iv) HHV-6 and HHV-7 are inhibited by the drug ganciclovir, increasing the safety of employing the vector in human gene therapy and vaccination.

(v) The vectors enable the expression of both cell surface-associated proteins as well as secreted proteins, thus ensuring a wide range of vaccination targets.

In addition to the inherent safety factors of HHV-6A and HHV-7 described above, defective virus vectors were now generated that do not damage the host cell, yet are capable of efficient expression of selected transgenes in lymphocytes and dendritic cells known to have the capabilities of efficient MHC based antigen presentation.

According to one aspect of the invention, there is provided a lymphotropic vector, optionally carrying one foreign gene or more, wherein administration of said vector to a mammal results in an immune response.

According to another aspect of the invention, there is provided a lymphotropic vector comprising at least one gene, and optionally carrying one foreign gene or more, wherein administration of said vector to a mammal results in an immune response against a product of any one of the genes in said vector.

According to yet another aspect of the invention, there is provided a lymphotropic vector carrying at least one foreign gene, wherein administration of said vector to a mammal results in an immune response against the product of said foreign gene.

In one embodiment, said foreign gene encodes a membrane-associated protein product. In another embodiment, the foreign gene is a soluble protein, which may be secreted outside of the cell.

In one embodiment, the lymphotropic vector is a recombinant DNA vector comprising:

(a) a DNA sequence derived from HHV-6 or HHV-7, said DNA sequence comprising an origin of replication, a promoter sequence which induces expression of at least one nucleic acid sequence product in a lymphocyte cell host and a cleavage and packaging signal; and

(b) optionally a foreign nucleic acid sequence capable of being expressed in said lymphocyte;

wherein administration of said vector to a mammal results in an immune response.

In a preferred embodiment, the vector is not capable of autonomous replication in a mammalian host cell, i.e. it is comprised of a replication-defective

genome. In a most preferred embodiment, said replication-defective genomes are concatameric, thus enabling reiterated repeats of the gene of interest, and hence strong and efficient expression of the gene product in a host cell.

In another embodiment, the lymphotropic vector is either amplicon-6 (see Example 1) or Tamplicon-7. Since these vectors are replication defective, they can infect the cells of the immune system, do the work of gene expression, arouse immune response and then leave the scene upon lymphocyte divisions. The amplicon vectors may be propagated for elongated periods of time only with constant addition of a helper virus, such as HHV-6 or HHV-7. In a preferred embodiment, the helper virus is HHV-6A .

Examples of foreign nucleic acid sequences which may be inserted into the vector of the invention are GFP, gp160, REV, gD or gDsec, Muc-1 breast cancer antigen, adjuvant genes to fortify the immune response, IL2, cytokines and chemokines.

According to another aspect of the invention, there is provided a method for eliciting an immune response in a mammal, said method comprising the steps of:

(a) providing a lymphotropic vector, optionally carrying a foreign nucleic acid sequence of interest;

(b) introducing said vector into the body of said mammal;

wherein said introduction results in an immune response in said mammal.

According to yet another aspect of the invention, there is provided a method for eliciting in a mammal an immune response against a protein product of nucleic acid sequence of interest, said method comprising the steps of:

(a) providing a lymphotropic vector carrying a nucleic acid sequence of interest; and

(b) introducing said vector into the body of said mammal.

wherein said introduction results in an immune response against said product of said nucleic acid sequence of interest in said mammal.

In a preferred embodiment of said method, said lymphotropic vector is derived from HHV-6 or HHV-7. Most preferably, said lymphotropic vector is either amplicon-6 or Tamplicon-7.

The lymphotropic vector in the methods described above may be according to any one of the embodiments described above. The lymphotropic vector may be introduced as pure DNA, or along with a helper virus, or in other forms as will be described in more detail below.

The vectors of the invention are being used as safe means for inducing an efficient immune response in a mammal. Therefore, according to another aspect of the invention, there is provided a pharmaceutical composition comprising at least on of the vectors of the invention and a pharmaceutically acceptable carrier.

In yet another aspect of the invention, there is provided a kit comprising at least one of the vectors of the invention and a pharmaceutically acceptable carrier, and instructions for use.

BRIEF DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Fig. 1 is a scheme showing the structure of Amplicon-6 and Tamplicon-7, the insertion site of a foreign gene, and the generation of defective genomes with multiple repeats of the amplicon sequence.

Fig. 2A-2D is a scheme showing the Tamplicon-7 vector system. **(A)** Tamplicon-7 containing the lytic replication origin (oriLyt) of HHV-7 and the packaging signals (pac) of HHV-7. **(B)** The Tamplicon-GFP construct containing also the Green Fluorescent Protein (GFP), driven by the Human Cytomegalovirus promoter (HCMV). **(C)** The formation of defective genomes, or concatameric amplicons in the presence of a helper virus. **(D)** Southern blot analysis of nuclear (nuc) and cytoplasmic (cyto) DNA preparations and DNA from purified virions prepared from the medium (med.). M denotes a 1-kb DNA marker ladder. pOrilyt is a construct that does not contain the packaging signals.

Fig. 3 is a schematic diagram of the propagation of cell associated and cell free amplicon 6 vectors containing EGFP (Enhanced Green Fluorescent Protein).

Fig. 4A-4F shows fluorescent microscope photographs of J. JhanT cells that were transfected by electroporation with the vector Amp-6 EGFP (amplicon-6, containingn EGFP). **(A)** Passage 0 (P0) , the electroporated culture viewed 7 days p.t (post-transfection). **(B)** P0, infected – cells were transfected and 48 hrs later superinfected with the helper virus HHV-6A (U1102). They were viewed 7 days p.t. **(C)** Passage 1 (P1) – cultures which did not receive the HHV-6 helper virus were "passaged" by adding uninfected cells. **(D)** P1 – transfected/superinfected vectors were passaged to new, uninfected cells. Shown 1 week later. **(E)** P1 medium was filtered through 0.45μm filters allowing passage of virus but preventing cell passage and producing "cell free vectors". The filtered

medium was used to infect new cells, which were inspected week later **(F)** A scheme showing the structure of the amp-6-EGFP (pNF1194) plasmid.

Fig. 5A-5B shows the structure of amplicon-6 containing an intact gD gene driven by the HCMV promoter – Amp6-gD **(A)**, and amplicon-6 the gD gene wherein a 201 bp deletion of the transmembrane signal was introduced – Amp6-gDsec **(B)**.

Fig. 6 shows the expression of Amp6-gD mRNA in J. Jhan cells with and without infection with the HHV-6 helper virus. **Lanes 1 and 2** - Expression of gD mRNA from cells electroporated with Amp6-gD at 24 and 48hrs post transfection, assessed by reverse transcriptase (RT). **Lane 3** - Vero cells infected with HSV-1. **Lane 4** - plasmid DNA of the Amp6-gD vector. **Lanes 5 and 6** are identical to lanes 1 and 2 without the reverse transcriptase (RT) enzyme. **Lane 7** - DNA marker. **Lane 8** – same as lane 3, without RT.

Fig. 7 is a Western blot analysis of the expression of Amp6-gD and gDsec in J. JhanT cells. The blot was probed with anti-gD monoclonal antibodies (mAbs). **Lane 1** - proteins of HSV-1 infected Vero cells (Monkey kidney cells used for HSV propagation). **Lane 2** – marker. **Lane 3** – mock transfection. **Lane 4** – J. Jhan cells transfected with Amp6-gD, 7 days p.t. (post-transfection). **Lane 5** – J. Jhan cells transfected with Amp6-gDsec, 7 days p.t.

Fig. 8 is a Western blot analysis of the expression of Amp6-gD in J. JhanT cells, which were transfected with Amp6-gD with and without HHV-6 helper virus. The blot was probed with anti-gD mAbs. **Lane 1** - Vero cells infected with HSV-1. **Lane 2** – protein size marker. **Lane 3** – J. Jhan cells infected with helper virus, but not with Amp6-gD. **Lane 4** – J. Jhan cells infected with Amp6-gD, but not with helper virus. **Lane 5** – J. Jhan cells infected with both helper virus and Amp6-gD. **Lanes 6 and 7** – filtered medium of passage 0 cells without (lane 6) and with (lane 7) helper virus, was used to infect new cells. Seven days later, the proteins were analyzed. **Lanes 8 and 9** – Passage 1 of the cell-associated Amp6-gD without (lane 8) and with (lane 9) helper virus.

Fig. 9 is a Western blot analysis of the expression of Amp6-gDsec in J. JhanT cells, which were transfected with Amp6-gDsec with and without HHV-6 helper virus. The blot was probed with anti-gD mAbs. **Lane 1** - Vero cells infected with HSV-1. **Lane 2** –Protein size marker. **Lane 3** – J. Jhan cells infected with Amp6-gD, but not with helper virus. **Lane 4** – J. Jhan cells infected with both helper virus and Amp6-gD. **Lanes 5** – the medium of passage 0 Amp6-gDsec vector with helper virus was filtered, concentrated and used to infect new cells, generating cell passage 1. At the end of the infection proteins were prepared and analyzed in the Western blot probed with anti-gD antibodies. **Lanes 6 and 7** – Passage 1 of Amp6-gDsec propagated from the vectors with (lane 6) and without (lane 7) helper virus. **Lane 8** – Passage 2 of the Amp6-gDsec vector/superinfected cells.

Fig. 10 is a Western blot of trichloroacetic acid (TCA) precipitation of gDsec or gD from the medium of J. JhanT cells, which were transfected with Amp6-gDsec or Amp6-gD, with and without HHV-6 helper virus. The blot was probed with anti-gD mAbs. **Lane 1** - Vero cells infected with HSV-1. **Lane 2** – protein size marker. **Lane 3** – Passage 0 (P0) of J. Jhan cells transfected with Amp6-gDsec, 48 hrs post-transfection (p.t.), without helper virus. **Lanes 4 and 5** – TCA precipitated medium of the P0 gDsec culture, 48 hrs (lane 4) or 7 days (lane 5) p.t., without helper virus. **Lane 6** – TCA precipitated medium of the P0 gDsec culture, 7 days post-transfection, with helper virus. **Lanes 7-10** - transfections with Amp6-gD including electroporated cells (lane 7) and TCA precipitated medium of the P0 electroporated Amp6-gD, 2 and 7 days p.t. without helper virus (lanes 8 and 9) and with helper virus (lane10). The TCA precipitated proteins appeared to be smaller than the non-TCA precipitated proteins, indicating breakage.

Fig. 11A-11F shows confocal microscope images of J. Jhan cells infected with both HHV-6 helper virus and Amp6-gD. The cells were stained with the H170 anti-HSV-gD antibody. Each part of the figure is composed of: upper left-fluorescent photo, upper right- differential interactions contrast (Nomarsky)

photo, and the lower left- superposition of the fluorescent and Nomarsky photo. (A) HHV6A (U1102) infected J. Jhan cells. (B – F) Representative images of J. Jhan cells transfected with Amp6-gD and superinfected with HHV6A (U1102).

Fig 12A-12F shows confocal microscope images of J. Jhan cells infected with both HHV-6 helper virus and with Amp6-gDsec. The cells were stained with the H170 anti-HSV-gD antibody. A – F - Representative images of J. Jhan cells that are transfected with Amp6-gDsec, and superinfected with HHV6A (U1102). Each part of the figure (e.g. A) is composed of: upper left - fluorescent photo, upper right- differential interactions contrast (Nomarsky) photo, and lower left- superposition of the fluorescent and Nomarsky photos.

Fig. 13 is a schematic representation of Amplicon-6 vectors carrying the HIV-1 gp160 gene and both gp160 and REV genes.

Fig. 14 is a Western blot analysis of amplicon-6-gp160 expression in 293 cells. The blot was probed using anti-gp120 1A8 mAbs. **Lane 1** – control, mock transfected cells. **Lane 2** – cells transfected with Amp6-gp160. **Lanes 3 and 4** – cells transfected with mixtures of both amplicon-6-gp160 and amplicon-6-REV clone a (lane 3) and clone b (lane 4). **Lanes 5 and 6** – cells transfected with clone 9 (lane 5) or clone 15 (lane 6) of Amp6-gp160-REV. **Lane 7** - purified gp120 as positive control.

Fig. 15 shows a Western blot analysis of Amp6-gp160-REV expression in J. Jhan cells with and without HHV-6 helper virus. The blot was probed using the anti-gp120 1A8 mAb. **Lane 1** – pure gp160 as positive control. **Lane 2** – protein size marker. **Lane 3** – untransfected cells. **Lane 4** – cells infected with helper virus but no amplicon vector. **Lanes 5 and 6** – P0 cells transfected with amplicon-6-gp160-REV without (lane 5) and with (lane 6) helper virus. **Lanes 7 and 8** – filtered medium of P0 Amp6-gp160-REV transfection without (lane 7) and with (lane 8) helper virus. The filtrate was passaged to new cells which were

tested 7 days later. **Lanes 9 and 10** - passage 1 of cell-associated Amp6-gp160-REV without (lane 9) and with (lane 10) helper virus.

Fig. 16 shows a Western analysis of propagated Amp6-gp160-REV in J. Jhan cells. The blot was probed using anti-gp120 1A8 mAb. **Lane 1** – untransfected cells. **Lane 2** – cells infected with helper virus but without the amplicon vector. **Lanes 3 and 4** – P0 cells transfected with Amp6-gp160-REV without (lane 3) or with (lane 4) helper virus. **Lane 5** – passage 1 of cell associated Amp6-gp160-REV with helper virus. **Lane 6** – passage 2 of cell associated Amp6-gp160-REV with helper virus. **Lane 7** – filtered P1 Amp6-gp160-REV with helper virus was passaged to new cells, generating P2 infection cultures, which were assayed 7 days later. Lane 8 – the medium of P2 cells was filtered and passaged to new cells which were assayed 7 days later.

Fig. 17A-17B shows a confocal microscope analysis of J. Jhan cells transfected with Amp6-gp160-REV and superinfected with the U1102 helper virus. Cells were stained using the anti-gp120 1A8 mAb. Each part of the figure is composed of: upper left – fluorescent image, upper right – Nomarsky imaging, lower left – superposition of the two images. (A) Cells infected only with helper virus. (B) Cells transfected with Amp6-gp160-REV and superinfected with the U1102 helper virus.

Fig. 18A-F shows a confocal microscope analysis of J. Jhan cells transfected with Amp6-gp160-REV and superinfected with the U1102 helper virus. Cells were stained with the CG10 anti-gp120-CD4 complex mAb. Each part of the figure is composed of: upper left – fluorescent image, upper right – Nomarsky imaging, lower left – superposition of the two images.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The composite amplicon vectors of the invention consist of two components: (i)defective genomes with multiple reiterations of amplicon units,

each containing the DNA replication origin and packaging signals, as well as the selected transgene(s). (ii)an adequate helper virus which provides the DNA replication and packaging functions and the structural. In the presence of the helper virus the amplicons replicate by the rolling circle mechanism, producing large concatamers of the input amplicons with the signals pac-1 and pac-2, located at the junctions between repeats. The concatamers are cleaved 29-35 bp away from the pac-1 signal and 40-45 bp away from the pac-2 signals, located at approximately "headfull" or full length genomes, resulting in defective genomes of overall size 135-150 kb made of multiple reiterations of amplicon units (Romi et al., 1999). The defective viruses follow their nondefective helper viruses in their cell tropism and ability to infect dividing as well as non-dividing cells. HHV-6 was shown to infect mature T lymphocytes, lymph nodes, macrophages and monocytes, dendritic cells, kidney tubule endothelial cells as well as CNS tissues.

The defective amplicon virus vectors of the invention are capable of efficient expression of selected transgenes in lymphocytes and dendritic cells known to have the capabilities of efficient MHC based antigen presentation. As described below the system was assayed employing the GPF marker gene, the gD and gDsec genes to inhibit facial and genital herpes infections and the HIV glycoprotein gp160, towards development of an AIDS vaccine. The amplicon–6 vectors are expressed most efficiently in T cells (see below). Immunization experiments using purified defective virus DNAs and virus vectors with or without helper viruses are currently ongoing. Vaccination is being tested in mice for both humoral and cellular immunization. In addition to viral vaccination employing herpesvirus genes and targeting HSV facial and genital disease, potential herpesvirus vectors could also target Varicella Zoster Virus (causing chicken pox in the young but also most painful Zoster infections in elderly people), cytomegalovirus (with most grave consequences in pregnant women and also in bone marrow and other transplantation) and other viruses. This may be done be inserting the relevant genes of interest into the amplicon

vectors of the invention, and introducing them to the immune system by means which will be described below. Vaccination efficiencies can be improved significantly with the amplicon-6 vector relative to existing genetic vaccination systems, due to the sequence reiterations of the vectors of the invention, which give rise to a high level of expression of the DNA sequence(s) of interest.

HSV vaccines as live virus, attenuated virus as well subunit vaccines have been previously reported by others, without significant success. The transport of the gD gene into lymphocytes out of the virus grown in epithelial and mucosal cells is expected to significantly increase efficiency, inasmuch as the natural HSV contains functions that are known to escape and evade the immune system. Furthermore expression of multiple copies per cell is most efficient and results in overproduction of the selected DNA sequences. Finally, an additional extension for the potential use of amplicon–6 vectors for efficient antigen presentation in lymphocytes includes cancer vaccination employing proteins which have abnormally high expression in malignant cells and tissues e.g., the muc-1 protein in breast cancer, and the prostate specific antigen (psa) for prostate cancers. Greater efficiency in vaccine production is predicted.

Examples of the lymphotropic vaccination vectors of the invention are:

(One) human herpes virus 6 (HHV-6);

(Two) a mutant of HHV-6 capable of binding to the CD46 receptor;

(Three) a virus particle of the virus of (a) or (b);

(Four) amplicon-6;

(Five) Tamplicon-7;

(Six) Fragments of (d) or (e) and combinations thereof; and

(Seven) any combination of the agents under (a) to (g).

In addition to the DNA sequences derived from HHV-6 or HHV-7, the vectors of the invention comprise an origin of DNA replication, a promoter sequence capable of inducing expression in a lymphatic host cell of a downstream

nucleic acid sequence and a cleavage and packaging signal. The vectors may optionally comprise also a foreign nucleic acid sequence downstream to an expression control of said promoter sequence.

For therapeutic use, said lymphotropic vector is incorporated into a delivery vehicle. A large number of vehicles are available for the delivery of genetic material into cells, delivery vehicle which are viral-derived particles are generally preferred in view of the specificity of such particles to certain cells which facilitate the targeting of the genetic material to such cells. Seeing that the lymphotropic vector of the invention is derived from HHV-6 or HHV-7, the preferred viral particle for use as a delivery vehicle is derived from these two respective viruses. There is some evidence that HHV-7 may activate HHV-6 replication (Katsafanes *et al.*, 1996), and accordingly, it is also possible in accordance with the invention to use an HHV-7 particle as a delivery vehicle for an HHV-6 derived lymphatic vector.

HHV-6 or HHV-7 particles have an affinity to specific cell types. The HHV-7, binds to the CD4 receptor and accordingly the particle derived from the HHV-7 is particularly useful for the delivery of said lymphotropic vector to $CD4^+$ cells. The HHV-6 particles have an affinity to a variety of cells and mainly to both $CD4^+$ and $CD8^+$ cells, as well as to some other lymphatic cells, e.g. EBV infected B-cells, and may thus be useful for the targeting of said lymphotropic vector to such cells, as well as to dendritic cells which are the most efficient antigen presenting cells.

The preferred delivery vehicle in accordance with the present invention, is a member selected from the group consisting of:

(One) an HHV-6 or HHV-7 particle;

(Two) a mutant HHV-6 or mutant HHV-7 particle capable of infecting lymphatic cells and delivering its content of DNA to such cells;

(Three) a chemically modified particle of (a) or (b) essentially retaining the ability to infect lymphatic cells; and

(Four) any combination of (a), (b) or (c).

Two kinds of vectors are provided by the present invention: a vector which is capable of autonomous replication (hereinafter: *"ARV"* (autonomously replicating vector)); a vector which is not capable of self replication (hereinafter: *"amplicon"*). While an ARV can be administered by itself, an amplicon is administered together with a helper virus which provides the transactivation factors for replication of the amplicon. A helper virus is typically a self-replicating HHV-6 or HHV-7. The choice of the helper virus may typically be based on the nature of the amplicon: in case of an amplicon derived from HHV–6, a self-replicating HHV-6 will typically be used, preferably HHV-6A. In the case of a Tamplicon derived from HHV-7, a self-replicating HHV-7 will typically be used. As pointed out above, a self-replicating HHV-7 may be used as a helper virus for an HHV-6 derived amplicon. Alternatively, HHV-6A may be used as a helper virus for an HHV-7 derived Tamplicon.

As already pointed out above, HHV-6A and HHV-7 has no known abundant pathology and therefore their use as helper viruses is generally preferred where possible over the use of HHV-6B. However, use of HHV-7 is limited in view of the fact that it infects primarily $CD4^+$ cells and accordingly use of HHV-6A is at times preferred. In case use is made of the HHV-6, measures should be taken to neutralize this virus after such period of time. Alternatively, a mutant HHV-6A may be used, the expression of which may be controlled by changes in various factors such as, a change in temperature (i.e. a temperature sensitive mutant).

The helper virus functions can be provided by superinfecting virus, or by co-transfection with large DNA clones, or by first cloning the entire genome lacking packaging signals in large bac vectors, then placing all genes in a cell line on top of which the defective genomes with multiple copies of the

amplicons can be placed and used for production of pure defective vectors. Potentially the pure vectors can be also be used for vaccination.

Mutant viruses may be obtained by standard methods. An example of a mutant is such which lost its ability to replicate by itself in a host cell. Another type of mutant may, for example, be such which has a higher affinity to binding to the CD4 receptor than the native strain.

A particle of the virus may be obtained by various standard methods which are known in the art. Various polypeptides, are obtainable either by chemical methods or by methods of genetic engineering, namely, by cloning and expressing a gene coding for the polypeptide. Such a polypeptide is typically a portion of the capsule which determines the binding affinity of the capsule to the $CD4^+$ receptor. Polypeptides produced by means of genetic engineering avery often obtained as fusion proteins of the desired polypeptide with another protein or peptide component. Such fusion proteins, defined under (e), may also be useful at times as said CD4-ligand.

Derivatives may be obtained by various standard chemical or biochemical methods, or by methods of genetic engineering, such methods being generally known *per se*.

The specific regimen for vaccination can be determined for each antigen by routine methods known to those skilled in the art. In each case, the vaccination regimen should ensure an effective amount of antigen will be presented to the immune system of the subject. For some antigens, a high *in vivo* level of the vaccination agent (i.e. the lymphotropic vaccination vector) in the blood may be desirable. In such cases, the use of an ARV or of a helper virus may be preferable.

In other cases, it may be desirable to stop the expression of the lymphotropic vector at a desired point of time. In such case, it is preferable to use a helper virus or even a lymphotropic vector with a carrier vehicle, but without a helper virus.

As mentioned above, the invention provides pharmaceutical compositions comprising any one of the vectors of the inventions, along with a pharmaceutically acceptable carrier. As described above, a pharmaceutically acceptabel carrier is any inert, non-toxic material, which does not react with the vectors of the invention. The carriers may also refer to substances added to pharmaceutical compositions to give a form or consistency to the composition when given in a specific form, e.g. in a form suitable for injection, spray, aromatic powder etc. The carriers may also be substances for providing the composition with stability (e.g. preservatives).

The choice of carrier will be determined in part by the particular vector, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of the pharmaceutical composition of the present invention. A preferred formulation is that suitable for parenteral administration, for example subcutaneous, intravenous, intraperitoneal or intramuscular, either systemically or locally. The requirements for effective pharmaceutical carriers for injectable compositions are well known to those of ordinary skill in the art. See, for example, *Pharmaceutics and Pharmacy Practice*, J.B. Lippincott Co., Philadelphia, Pa., Banker and Chalmers, eds., pages 238-250 (1982), and ASHP Handbook on Injectable Drugs, Toissel, 4th ed., pages 622-630 (1986). It may also be administered by intravenous infusion.

As an example, for the preparation of a pharmaceutical composition suitable for parenteral administration, e.g. intravenously by *iv drip* or *infusion*, dosages in the range of from 1 mg to 10 mg per kg body weight (the lower concentrations are preferable). However, the pharmaceutical composition for treatment in the method of the invention is not limited to these dosages, and other appropriate dosages are well within the competence of the skilled artisan to select.

Carriers suitable for injectable formulations of the compositions of the invention may include, without being limited thereto, vegetable oils, dimethylacetamide, dimethylformamide, ethyl lactate, ethyl carbonate, isopropyl myristate, ethanol, polyols (glycerol, propylene glycol, liquid polyethylene

glycol, and the like). For intravenous injections, water-soluble versions of the therapeutic agent may be administered by the drip method, whereby a pharmaceutical formulation containing a vector and a pharmaceutically acceptable carrier is infused. Specific pharmaceutically acceptable carriers may include, for example, 5% dextrose, 0.9% saline, Ringer's solution or other suitable excipients. Intramuscular preparations, e.g., a sterile formulation of a suitable soluble salt form of the antibody, can be dissolved and administered in a pharmaceutical excipient such as Water-for-Injection, 0.9% saline, or 5% glucose solution.

As should be appreciated, the pharmaceutical composition may be in the form of a medical formulation kit, together with at least one type of medical carrier or diluent.

Materials and Methods

Plasmids

pSVIIIgp160 – clone 92HT593.1, gene bank accession no. U08444, contains the gp160 gene of HIV-1 subtype B, was received from the NIH AIDS Research and Reference Reagent Program.

pSV-REV – contains the REV cDNA gene of F12-HIV-1 subtype B. (gift of Prof. Jonathan Gershoni and Dr. Galina Denisova, Tel Aviv University).

Antibodies

Primary antibodies:

H-170 – A mouse anti-gD IgG, recognizes linear epitope at the N-terminal of HSV-1 and HSV-2 gD (gift of Dr. Lenore Pereira, Department of Stomatology, school of Dentistry, University of California, San-Francisco, USA).

1A8 – A mouse anti-gp120 IgG, recognizes linear epitope at the N-terminal of HIV-1 gp120. (gift of Prof. Jonathan Gershoni, Tel Aviv University).

cg10 – A mouse anti-gp120-CD4 complex IgG antibody (gift of Prof. Jonathan Gershoni, Tel Aviv University).

Secondary antibodies:

Goat Anti-Mouse IgG antibody- $Cy^{TM}3$-conjugated conjugated affinipure Goat anti-mouse IgG $F(ab')_2$, bought from jackson immunoResearch laboratories. (Code no. 115-166-072, Lot: 40709).

Goat Anti-Mouse IgG antibody- FITC-conjugated affinipure Goat anti-mouse IgG (H+L), bought from Jackson ImmunoResearch laboratories. (Code no. 115-095-062).

Goat anti-mouse IgG antibody- peroxidase-conjugated affinipure Goat anti-mouse IgG (H+L), bought from Jackson ImmunoResearch laboratories. (Code no. 115-035-146, Lot no. 51633).

Cell cultures and viruses

Cell Cultures

Two human CD4+ T-lymphocytes cell lines were used: **J. Jhan cells** - derived from Jurkat T cells, and **Sup-T1 cells** - derived from a non-Hodgkin's T-cell lymphoma patient (ATCC CRL-1942). Both cell lines were propagated in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine (Biological Industries), and 50µl from 50mg/ml of gentamicin stock. These cell lines grow in suspension. **293T cells** are propagated in DMEM medium, supplemented with 10% (FCS), 2mM L-glutamine (Biological Industries), and penicillin (20U/ml), streptomycin (20µg/ml), nystatin (2.5U/ml): (PEN-STREP-NISTATIN, Biological industries). All cell cultures were incubated at 37°C in a humidified 5% CO_2 incubator.

Viruses and *in vitro* infection

The HHV-6A strain U1102 was obtained from Dr. Robert Honess and propagated in J. Jhan or in the cell line SUP-T1 cell lines. The viruses were propagated by cocultivation of infected cells with fresh uninfected cells (1:1

ratio). Uninfected cells were incubated with infected cells for 2 hr at 37°C in a humidified 5% CO_2 incubator, in a concentrated aliquots of volume <1 ml for absorption of the virus. After the absorption, the infected cells were diluted into RPMI 1640 medium containing 10% fetal calf serum. Infection was assessed by the appearance of a cytopathic effect characterized by marked enlargement of infected cells and formation of syncytia.

Extraction of total infected cell DNA

Total DNA was extracted from infected and non-infected cultures by using the EZ-DNA genomic isolation kit (Biological Industries co.), according to the supplier's protocol (based on the Guanidinium Isothiocynate reagent).

Extraction of total infected cell RNA

Total RNA was extracted from infected and non-infected cultures by using the EZ-RNA Total RNA Isolation Sample kit (Biological Industries co.), according to the supplier's protocol (based on the Guanidinium Isothiocynate reagent for denaturation, phenol and chloroform for extraction and protein removal).

Protein assay from transfected cells

Transfection–Infection (superinfection) assay –

24h before electroporation the J. Jhan cell were passaged, and then washed twice in PBS (without calcium and magnesium). The washed cells were resuspended in RPMI-1640 medium at 1×10^7 cells/ml. 0.4ml of the cells were mixed with 50µl of purified plasmid DNA in 4mm gap disposable cuvette (BTX P/N 640), and then electroporated by one pulse of 250 V, 24msec (Electro cell manipulator ECM 395). The electroporated cells were incubated for 10 min on ice and then transferrto 5ml of RPMI 1640 medium supplemented with at 10% fetal calf serum and 50 µl/ml gentamicin at a final dilution of 4×10^6 cells/ml. 24h - 48h after electroporation, the cells were

infected with concentrated aliquots of the HHV-6 U1102 strain infected cells. Viral cytopathic effects peaked usually 5 to 8 days after infection, and the electroporated/infected cells were harvested for protein extraction.

Protein extraction

The superinfected cells were washed twice with PBS without calcium and magnesium (Biological industries), then lysed by adding 200µl of 4 °C lysis buffer. The lysed cells were rotated at 4 °C for 1h, and centrifuged in an eppendorf centrifuge 20' – 30' at 14000rpm. The supernatant was collected into new eppendorf tubes and frozen at -70 °C. Aliquot of the extracted proteins were measured to determine the protein concentration, by the Bradford method, using a 96 well ELISA reader at 595nm wavelength. BSA was used for calibration.

Protein precipitation by TCA

To precipitate the proteins secreted to the medium, 24h – 48h before precipitation the cells medium was replaced to Bio-Ram-1 medium (protein free). The cells were pelleted by centrifugation 5' at 2000 RPM. The medium was filtrated through 0.45µm filter. To each 0.5ml fraction (in eppendorf tube) 1µg of BSA was added as a carrier. 125µl of 50% TCA was added and mixed, to give final concentration of 10% TCA. The mixture was incubated 10' at -20°C, and then spun in a microcentrifuge at 4°C, top speed for 20-30min. The supernatant was carefully removed and the pellet was resuspended in 12-20µl of 1 × loading buffer with β-mercaptoethanol. If the color was yellow, (indicating acidity), 0.5-7µl of 1M Tris pH 8.0, were added till the color turned blue.

Electrophoresis of protein molecules

The electroporated / infected cells were harvested and lysed in 50mM Tris HCL (pH7.5), 150mM NaCl, 0.5% NP-40 and protease inhibitors

(Complete protease inhibitor, Roche). Protein samples were first denatured by 5' boiling and β-mercaptoethanol (Sigma), in the loading buffer, and then were loaded on 8-12% Tris-Glycine SDS-polyacrylamide gels, in the Bio-Rad running device, employing running buffer at constant current of 40MA per gel. A molecular weight protein marker was used. The gel was transferred to nitrocellulose membrane (Schleicher & Schuell), washed 3 times in ddH_2O and stained with a gel code blue stain reagent.

Western blotting

The proteins that ran on the Tris-Glycine SDS-polyacrylamide gel were transferred to nitrocellulose membrane by attaching the gel to the membrane and by pressing with Whatman paper and Dacron sponges from both sides. The cassette was placed inside the transfer device (Bio-Rad), in transfer buffer with an ice vial, at a constant voltage of 60V for 2h-3h.

Immunoblotting

The nitrocellulose membrane with the transferred proteins, was blocked by 5% milk in TTBS for 1h at R.T. Then, rinsed once briefly with TTBS and incubation with the primary antibody diluted between 1:200 to 1:5000, in TTBS with 1% BSA and 0.05% Sodium Azid (NaN_3). The membrane was incubated with the primary antibody for 2h R.T. The membrane was washed 4 times 5' each with TTBS, and then incubated with the secondary antibody Goat anti-mouse IgG peroxidase-conjugated affinipure diluted 1:5000 to 1:25000 in 5% milk in TTBS , (Jackson Immunoresearch Laboratories). The membrane was reacted with the secondary antibody for 45' – 60' at R.T, and washed 4 times 5' each with TTBS. Enhanced chemiluminescence (ECL) mixture (SuperSignal West Pico Chemiluminescent Substrate (ECL) – Pierce), was added to interact with the horseradish peroxidase (HRP), the tag on the secondary antibody, causing light emission, detected on Scientific imaging X-OMAT Kodak film.

Detection of GFP in lymphocytes

300µl samples of cells were washed once with PBS and resuspended in 1/10 volume of PBS. The concentrated cells were placed on glass slides that were coated with poly-L-lysine (1mg/ml). The cells were fixed with 4% paraformaldehyde for 15' - 20' R.T, and washed with PBS. Then Galvanol was added and covered with cover slip. The fluorescent cells were visualized using fluorescence Axioskop microscope (Carl Zeiss, NY) and camera photographs were taken using 200ASA color films (MC-100 camera).

Immunofluorescence studies in lymphocytes

300µl samples of cells were washed once with PBS and resuspended in 1/10 volume of PBS. The concentrated cells were placed on glass slides that were coated with poly-L-lysine (1mg/ml). The cells were fixed with 4% paraformaldehyde for 15' - 20' R.T, washed 1' 3 times with PBS, before the addition of 0.1% TritonX-100 and incubated for 10' at R.T. For immunofluorescense detection inside the cell, this step was optional. The TritonX-100 was washed twice for 5', and the cells were then exposed for 30' at R.T to 20% fetal calf serum in PBS for blocking. The cells were then incubated for 30' at R.T with the primary antibody at 1:200 dilution, rinsed 3 times with PBS at R.T for 10' with gentle shaking. Secondary antibody, (1:500 Goat anti-mouse IgG Rodamine-conjugated) was added for 30' at R.T. Cells were then washed with PBS 3 times for 10' at R.T, with gentle shaking. At the end Galvanol mounting reagent at 100 mg/ml Mowiol (Calbiochem, LaJolla, CA) was added and cells were covered with cover slip. The cells were viewed with an Axiovert 135M confocal microscope (Carl Zeiss, NY) equipped with an argon-krypton laser using a 100X objective lens; excitations were at 488 and 568 nm. Contrast and intensity for each image were manipulated uniformly using Adobe Photoshop software.

PCR amplification

Generally 200ng - 1μg DNA as a template or DNA from bacteria colonies were taken by picking. The prepared reaction mixture contained 10μM of each primer, 2.5U *Taq* polymerase with standard buffer conditions ($MgCl_2$, 1.5 mM final concentration), and 10mM deoxnucleotide triphosphate mixture (2.5 mM each), in a total of 50μl per reaction. The PCR amplification reaction profile was usually one cycle of 5' at 94°C followed by thirty cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C - 65°C (depending on the primer's annealing temperature), and extension for 1 - 3 min at 72°C (depending on the amplified section length). Following the 30 cycles, an additional 10' at 72°C needed for complete polymerization, and cooling to 10°C till the samples were carried out from the PCR. The PCR amplification reactions were done in a DNA thermal cycler (TechGene, Techne).The amplified products were then electrophoresed in 1% agarose gels with ethidium bromide staining. The products were gel extracted and cloned into the appropriate vector as detailed in "Results".

Sequences of DNA oligonucleotide primers used:

gD sense:	5'- CAG CTT CAC GAC CGG TAG GTC TCT TTT GTG TGG TGC -3'
gD anti sense:	5'-GAT ACT AGC CTG ATC AGG GGT ATC TAG TAA ACA AGG -3'
gD sec anti sense:	5' ACT AGC CTG ATC ACT AGG CGT CCT GGA TCG ACG G 3'
gD sequence 301	5'- GAG GCC CCC CAG ATT G -3'
gD sequence 639	5'- CTG TAA GTA CGC CCT CC -3'
gD sequence 3151	5'- GTA ACA ACT CCG CCC CAT -3'
gp160 short sense	5'- GTG GCA ATG AGA GTG AAG -3'
gp160 short anti sense	5'- CTA TAG CAA AGC CCT TTC C -3'

gp160 long sense	5'-CAG CTA CCG CTG GCC GGC CAG GCC TGT GCA GCG TAC GGT GGC AAT GAG AGT GAA GGA G -3'
gp160 long anti sense	5'- GAT ACT GAT CAG GCC ATT CAG GCC TTC GAA CGT ACG CTA TAG CAA AGC CCT TTC CAA AC -3'
gp160 sequence 402	5'- GGA GAA TAG TAC TAA TGC C -3'
gp160 sequence 1024	5'- GAC ACC TTA GGA CAG ATA G -3'
gp160 sequence 1705	5'- CAG CTC CAG GCA AGA ATC -3'
gp160 sequence 2356	5'- GCC CTC AAG TAT TGG TGG -3'
REV HXB2 s 5970	5'- GGA TTG TGG AAC TTC TGG -3'
REV HXB2 as 6030	5'- GCT TGA TGA GTC TGA CTG -3'
Rev promoter 603	5'- GTT CGG CTG CGG CGA G -3'
CMVp sequence	5'- GTA CGC GGG GCT AGA GCG -3'
CMVp end seq	5'- GTA ACA ACT CCG CCC CAT -3'

The DNA oligonucleotide primers were synthesized using a DNA synthesizer (Sigma).

Reverse Transcriptase (RT)-PCR reaction

Samples of total RNA from infected and uninfected cells were used for RT reaction (with Expand Reverse Transcriptase kit, Roche). Reaction included 5μg of total RNA, 100pmols oOligo $(dT)_{15}$ and sterile RNase-free H_2O up to 31μl. The mixture was incubated 10' at 65°C and placed on ice for 2'. The other reagents were added to the same tube as follows:

4μl of 5*Expend reverse transcriptase buffer

2μl of 100mM DTT

8μl of dNTPs 10mM each

20 units of RNase inhibitor

50 units of Expend Reverse transcriptase

The reaction was incubated for 1h 42°C and then for 5' at 95°C.

The cDNA (cooled to 4°C and stored at -20°C), was used as a template (1μl - 5μl), for regular PCR amplification reaction.

Plasmid construction and purification.

The GFP plasmid (pEGFP-C3) contained a multi cloning site linker at the C-terminus of the GFP gene, designed for fusion proteins. The linker was removed and the coding region was cloned in Bluescript – SK.EGFP (pNF1193). Finally, a fragment that contained both the cleavage and packaging signals and the origin of replication (r-pac/orilyt fragment) was cloned in pNF1193 and the final construct was designated Amplicon-6.EGFP (pNF1194) (Fig 4F). Amplicon 6-gD (pNF 1215) – and the Amplicon- 6-gDsec (pNF1219) contain the gD sequences of HSV-1 (F). The Amp6-gDsec contains the first 327 amino acids (aa) of the gD1 gene and lacks 67 aa at the carboxy terminus, which include the transmembrane region (TMR). The gD1 constructs have the human cytomegalovirus promoter and the SV40 polyadenylation signal. Plasmid DNAs were prepared in *E.coli* DH10B or the *E.coli* K12 GM2163 (DAM^- / DCM^-) bacteria, using the Nucleobond AX plasmid maxiprep kit of Macherey-Nagel, Germany.

Amp6-gp160 (pNF1220) contains the gp160 gene of HIV subtype B from pSVIIIgp160 – clone 92HT593.1, gene bank accession no. U08444, received from the NIH AIDS Research and Reference Reagent Program. The gene was coamplified from the clone by PCR and introduced into pNF1194 into AgeI-BclI sites, replacing the GFP. Amp6-gp160-Rev (pNF1221) contains, in addition to the gp160 gene, the REV cDNA of F12-HIV1 subtype B gp160 gene from pSV-REV clone. The REV gene was digested by XbaI and cloned into the parallel site in pNF1220, creating pNF1221. The amp6-gp160/amp6-gp160-REV constructs have the hCMV promoter and the SV40 polyadenylation signal. Plasmid DNA was prepared in E.coli DH10B or K12

GM2163 (DAM⁻/DCM⁻), using the Nucleobond AX plasmid maxiprep kit of Machery-Nagel Germany.

Transfection and superinfection

J.Jhan cells (400μL at concentratons of 10^7 cells/ml) in RPMI-1640 medium were electroporated with 50 μg purified plasmid DNA in 4mm gap disposable cuvettes (BTX P/N 640) by one pulse at 250 V, 24msec using the Electro cell manipulator ECM 395. The electroporated cells were incubated for 10 min on ice and then transferred to 5ml RPMI 1640 with 10% fetal calf serum and 50 μg/ml gentamicin at a final concentration of 4×10^6 cells/ml. At 24h-48h after electroporation the cells were superinfected with equal number of HHV-6 (U1102) fully infected cells. The cultures were harvested for further passaging and protein extraction 5 to 6 days later.

Confocal microscope analyses

To determine the location of expressed gD and gDsec and gp160 proteins in the cells, cell samples were concentrated, rinsed with PBS and placed on glass slides coated with poly-L-lysine (1mg/ml). After fixation with 4% paraformaldehyde, cultures were perforated with 0.1% TritonX-100 and rinsed with PBS. The slides were blocked with 20% fetal calf serum in PBS to reduce background, and then incubated for 30' with the appropriate antibodies. For gD and gDsec expression, slides were incubated with gD H170 antibody. For gp160 expression, slides were incubated for 30' with soluble CD4, followed by incubation with CG10 (mouse mAb IgG antibody) known to interact with the gp120-CD4 complex (gift of Prof. Gershoni, Tel-Aviv University). Slides were then incubated with secondary Cy3- or FITC-conjugated Goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). After PBS rinsing the slides were covered with coverslip and Galvanol mounting reagent [100 mg/ml Mowiol (Calbiochem, LaJolla, CA) prior to viewing in an Axiovert 135M confocal microscope (Carl Zeiss, NY) equipped with an argon-krypton laser using a 100X

objective lens. Excitation was at 488 and 568 nm. Contrast and intensity for each image were manipulated uniformly using Adobe Photoshop software.

The construction of amplicon-6. gD (pNF 1215) and amplicon-6 gD secreted (gDsec, pNF 1219).

The gD gene was derived by PCR of the BamHI-J fragment of HSV-1(F) (pNF 417). Two PCR primers containing oligonucleotide tails with the AgeI and BclI restriction enzyme sites were used: sense including the AgeI site, 5'- CAG CTT CAC GAC CGG TAG GTC TCT TTT GTG TGG TGC -3' and anti sense, including the BclI site: 5'- GAT ACT AGC CTG ATC AGG GGT ATC TAG TAA ACA AGG -3'. These sites match the AgeI and BclI bounding the CMV promoter and the SV40 poly A signal in amplicon 6- vector (pNF 1194, Fig. 2). The amplicon-6 gD constract (pNF 1215) was produced in *E.coli* K12 GM2163 (DAM^- / DCM^-) competent bacteria, because the BclI site is sensitive to methylation. The gD fragment, digested with AgeI and BclI restriction enzymes was ligated into the parallel sites of the pNF 1194 fragment without the GFP gene. The resultant colonies were screened by PCR picking. A number of the positive colonies were sequenced and compared with the original sequence, using NCBI/Blast. The matching plasmid amplicon-6.gD (pNF 1215) contains the intact gD gene.

To construct a secreted form of the gD gene, the transmembrane region of the gD gene was deleted by PCR resulting with a protein of 327 amino acids (a.a), instead of the original 394 a.a of the intact gD gene. The gD sec antisense including the BclI site was 5'- GAT ACT AGC CTG ATC AGG GGT ATC TAG TAA ACA AGG -3'. The gDsec fragment was digested with the AgeI and BclI restriction enzymes and ligated into the parallel sites on the pNF 1194 vector resulting in the Amp6-gDsec (pNF 1219).

EXAMPLES

Example 1: Preparation of the amplicon-6 and Tamplicon-7 vector system.

The plasmid pEGFP-C3 of Clontec contained a multi-cloning site linker at the C-terminus of the GFP gene, designed for fusion proteins. The linker was removed and the coding region was cloned in Bluescript – SK.EGFP (pNF1193). Then, a fragment containing both the cleavage and packaging signals and the origin of HHV-6 replication (r-pac/orilyt fragment) was cloned in pNF1193 and the final construct was designated Amp6-GFP (pNF1194). Note that Amp6-GFP has the CMV promoter between the PstI and AgeI, driving GFP, and the poly A bounded by the BclI. From this vector, the GFP gene may be replaced by any DNA sequence of interest, including the gD, gDsec and gp160 (see below). In each case the DNA sequence is obtained by PCR of a clone containing the desired DNA sequence, using primers, which carry in them the matching AgeI and BclI sites (see Materials and Methods).

Fig. 1 shows the amplicon-type vectors, amplicon-6 and Tamplicon-7. The amplicon-type vectors contain a DNA replication origin, the pac-1 and pac-2 packaging signals and a site to insert at least one DNA sequence (e.g. as in Romi et al., 1999). A rolling circle replication of the amplicon plasmid using enzymes and functions contributed by the helper virus yields defective virus genomes with multiple reiterations of the input amplicon plasmids. The concatameric genomes are packaged in virions contributed by the helper virus.

Example 2: Tamplicon-7 vector with the Green Fluorescent Protein (GFP) marker (Fig. 2A, 2B).

The 1.6-kb GFP gene was excised from the pEGFP plasmid (Clontech) and ligated to pNF1182 between the BamHI and PstI sites. The resulting plasmid was designated Tamplicon-7.GFP (pNF1196). Shown are restriction enzyme sites

for EcoRV (EV), SmaI (Sma), HindIII (H), HincII (HcII), BamHI (B), and PstI (P). The GFP gene is driven by the Human Cytomegalovirus (HCMV) promoter.

Two independent infected cultures were electroporated with Tamplicon-7 and a third culture was electroporated with pOrilyt-7. Nuclear (nuc) and cytoplasmic (cyto) DNA preparations and DNA from purified virions prepared from the medium (med.) were extracted from all three cultures, digested with XhoI and DpnI, and hwith a GFP probe. In the presence of helper virus, the amplicon replicates by the rolling circle mechanism, yielding long, defective genomes with concatameric amplicons (**Fig. 2C**). pOrilyt7 I unable to exit from the nucleus, hence, the pac signals (packaging signals) are needed to exit from the nucleus to the cytoplasm, and out into the medium. In contrast to pOrilyt7, Tamplicon-7-GFP has no problem exiting from the nucleus to the cytoplasm, and to the medium (**Fig. 2D**).

Example 3: Schematic diagram of cell-associated or cell-free amplicon vectors (Fig. 3).

J. Jhan human T cells were transfected by electroporation with the amplicon-6 vector containing the GFP marker (amp-6-GFP, see Materials and Methods). Two days later a portion of the cells were superinfected with the helper virus HHV-6A strain U1102. The transfected/superinfected cells (Passage 0) were examined for GFP expression and were passaged by addition of uninfected cells, producing passage 1 vectors. Vectors secreted into the medium at P0 were collected by filtration through 0.45 μm membranes, to produce cell-free virions, which were further passaged in uninfected cells, producing cell-free passage 1 vectors. The analysis and passaging were repeated by adding new cells, producing passage 2 viruses. The electroporated/superinfected cells could be passaged repeatedly.

As a control served the remaining electroporated cells which were not superinfected with a helper virus. These cultures were handled similarly to the superinfected cultures.

As can be seen, only in cultures superinfected with the helper virus was there successful propagation of the amplicon virions. The virions were present both as cell-associated viruses and as cell-free viruses.

Example 4: Expression of EGFP in T cells (Fig. 4).

Fig. 4A-4D shows fluorescent microscope micrographs of J. Jhan human T cells that were transfected with the plasmid Amp-6-GFP, prepared as above. Incubation continued with or without superinfection with the helper virus HHV-6A U1102, followed by passaging into new, uninfected cells.

Fig. 4A shows Passage 0 J. Jhan cells that were transfected with Amp-6-EGFP. Some of the cells express GFP as can be seen by the green fluorescence. Passage 0 cells were then superinfected with the helper virus (**Fig. 4B**), which in the production of large genomes containing multiple repeats of the GFP amplicon. Indeed, many more cells expressed GFP, and the expression level was higher, as can be seen by the increased intensity of fluorescence. Similar results were obtained by quantitative analysis using FACS (Fluorescence Activated Cell Sorter).

The vectors can be continuously passaged as "cell associated" (**Fig. 4D**) or "cell free vectors" (**Fig. 4E**), by the filtering the medium of the lymphocytes through a 0.45μm filters, then infecting new cells, repeatedly. Such Passage 1 cells also express GFP in high levels. In contrast the plasmid DNA with the GFP gene was expressed only in the transfected culture (**Fig. 4A**), but not be propagated repeatedly (**Fig. 4C**).

Fig. 4F shows a schematic representation of the Amp-6-GFP vector. GFP expression is driven by the Cytomegalovirus (CMV) promoter.

Example 5: Amplicon-6 vectors for suitable for vaccination against HSV glycoprotein D (Figs. 5 and 6).

HSV-1 and HSV-2 cause painful facial and genital infections in children and adults with life long latency and repeated recurrences. Complications of the

diseases are grave and include blindness and risk of fatal encephalitis in HSV infected children and adults. Furthermore, most severe brain infections associated with retardation in newborn infants are due to infection by a mother with active genital herpes during pregnancy and birth.

The HSV-1 gD gene product is a major glycoprotein present in structural virions and in infected cell surface. The gene product plays an important role in viral entry and fusion to the cell membrane.

The entry of HSV into cells is an elaborate process which involves the interactions of several HSV envelope glycoproteins with an array of different receptors. HSV entry occurs by fusion of the virion envelope with the plasma membrane, and results in release of tegumented nucleocapsid into the cytoplasm. All the human entry receptors interact physically with the virion envelope component gD. The current model for HSV entry envisions fusion of the virion envelope with plasma membranes following with cell membrane interactions of four glycoproteins, gD, gB and the heterodimer gH-gL components. Cells that express gD constitutively from a transgene become resistant to infection. Due to the important role of gD in viral entry into target cells, and because of its strong immunogenic properties gD has served as a common vaccination target.

The intact gD gene and a 201 bp deletion mutant lacking the transmembrane region (termed here as gD secreted, gDsec) were introduced into amplicon-6 (Amp6-gD and Amp6-gDsec respectively). Both genes are expressed under the control of the CMV promoter (**Fig. 5A-5B**).

The expression of mRNA encoding gD in cells which were transfected with the Amp6-gD vector was verified. The transfected cells were used for RT-PCR analyses, employing RNA prepared 24 and 48 hours post-transfection. The RT reaction produced a cDNA product which could be PCRed yielding the 1300 bp DNA product seen in **Fig. 6** (lanes 1 and 2). Control of HSV infected Vero cells also shows an identically sized RT PCR product (lane 3). Identical DNA was

obtained upon PCR of a plasmid containing Amp6-gD (lane 4). No RT PCR product was produced in experiment identical to lanes 1 and 2 (24 and 48 hrs post-transfection respectively), when the RT enzyme was omitted from the reaction (lanes 5,6). Likewise the HSV infected Vero cells did not yield a PCR product when the RT enzyme was omitted (lane 8).

Example 6: Expression of gD and gDsec in cells using amplicon-6 (Figs. 7-10).

Amp6-gD and Amp6-gDsec were used to electroporate J. Jhan T cells. As controls Vero cells infected with HSV-1 were used, as well as J. Jhan cells that underwent mock electroporation. Seven days post-transfection, the cells were harvested, and analyzed by Western blot, using anti-gD monoclonal antibodies (**Fig. 7**). As can be seen, the molecular weight of gDsec is smaller than that of gD, due to the deletion of the transmembrane domain.

In a second experiment, J. Jhan cells were transfected with Amp6-gD and Amp6-gDsec as above, and two days post-transfection (p.t.) a portion of the culture was superinfected with HHV-6A (U1102) helper virus and the rest served as control (Passage 0). Cells or the cell-culture medium were then passaged by addition of uninfected cells. Seven days later, cells were harvested at the various stages and analyzed by Western blot as above (**Fig. 8**). As can be seen by comparing lanes 4 and 5, expression of gD was significantly enhanced in the HHV-6 superinfected cultures. The addition of the helper virus (i.e. HHV-6) was also crucial for finding amp6-gD in the filtered medium which could be used to infect new cells (lanes 6, 7) and for expression of gD in passage 1 cells, as can be seen in lanes 8, 9: only superinfected cells expressed gD in passage 1 vector. Using the same techniques, Amp6-gD could be further passaged repeatedly.

The expression of gDsec was assayed in a similar manner (**Fig. 9**). Cells were transfected with Amp6-gDsec and after two days were either superinfected with helper virus, or served as control. Again, the addition of the helper virus

enhanced expression of gDsec (compare lanes 3 and 4 in **Fig. 9**), and the helper virus was necessary in order to achieve expression in passage 1 (lanes 5-7, **Fig. 9**). Superinfected passage 2 cells also express gDsec (lane 8, **Fig. 9**).

Since gDsec lacks a transmembrane domain, it is possible that it is secreted from the cells. Initially, the expression of gDsec in the medium could not be detected without the addition of the HHV-6 helper virus (**Fig. 9**). However, if proteins were precipitated from the medium by the addition of trichloroacetic acid (TCA), a very small amount gDsec could be detected in the medium (**Fig. 10**, lanes 4-5). The electrophoretic mobility wassimilar but not identical to the non-TCA precipitated cultures - the TCA precipitated proteins appeared higher in the gel. As can be seen, the addition of HHV-6 significantly increased the levels of gDsec in the medium. (**Fig. 10**, lane 6).

Example 7: Confocal analysis of the expression pattern of gD and gDsec (Figs. 11, 12).

When viewed in the confocal microscope the cells infected with the intact gD amplicon produced gD protein localized preferentially at the cell surface (**Fig. 11**), whereas the gDsec amplicon protein appeared to be dispersed in internal locations of the cells (**Fig 12**). Altogether the experimental data regarding gD and gDsec expression demonstrate the ability to have the protein expressed efficiently in the cell surface, within the cells and as a protein secreted outside the cells.

Example 8: Amplicon-6 vectors carrying the HIV-1 gp160 and REV (Figs. 13 - 17).

A second example of proteins which were chosen for amplicon-6 mediated expression in T cells towards vaccination, corresponds to the envelope (env) gp160 gene of HIV and the REV gene. The product of gp160 is a 160 KDa polyprotein precursor of the proteins glycoprotein 120 and gp41 present on the

virus envelope and infected cell membranes. Cleavage of gp160 is required for Env-induced fusion activity and virus infectivity. The protein, which is anchored to the cell membrane, contains determinants that interact with the CD4+ T cell receptor and co-receptors catalyzing the fusion between the viral envelope and the cell membrane. Most importantly, the env gp160 protein contains epitopes that elicit an immune response in AIDS patients. The REV gene is essential for the processing of the gp160 mRNA and its transport to polysomes.

Two Amp-6 vectors were prepared – one carrying only the env gene (Amp-6-gp160), and one carrying both the env and REV genes (Amp-6-gp160/REV, see Materials and Methods).

The gp160 and REV protein products were found to be expressed in the 293-monolayer cell line, when assayed by Western analysis (**Fig. 14**). More importantly the Amp-6-gp160/REV vector could be employed as an infectious virus to T cells, resulting in a very efficient expression of the gp160 gene, as shown by Western blots (**Figs. 15 and 16**). Confocal microscopy (**Fig. 17**) showed expression of the protein in cell membranes surrounding the cells. Furthermore the gp160/REV amplicon could be further passaged as cell free and cell associated vectors. Expression was significantly enhanced in cells superinfected with the helper virus. It can be concluded that the amplicon and Tamplicon vectors can be efficiently employed to express immunogenic genes in human T cells, including both secreted and membrane-associated proteins.

Example 9: DNA vaccination - the production of neutralizing antibodies and the ability to induce cellular immunization.

As a first test of the ability to elicit an immune response using the amplicon-type vectors, DNA vaccination is utilized. In DNA vaccination, the vectors are injected as naked DNA, and not as virion particles. DNA vaccination was shown to result in phagocytosis into macrophages and dendritic cells of the

immune system, and in production of neutralizing antibodies as well as induction of CTL activity and secretion of interferons. The concatameric nature of the vectors containing multiple repeats of the immunogenic gene is advantageous relative to DNA vaccination with a monomeric plasmid, as is done with other DNA vaccines.

In order to prepare purified amplicon-6 DNA constructs, Amp6-gD and Amp6-gp160/REV gD were purified from total cell DNA by digestion with restriction enzymes which digest the cell DNA and the helper virus DNA into small fragments but do not digest the large (150 kb) concatameric amplicon genomes, which are defective virus genomes. Large amounts of pure defective virus DNA were produced and are being tested in BalbC mice by DNA vaccination done by intradermal (subcutaneous) and intra-muscular injections.

Specifically, 10 μg DNA per mouse, were injected into the tail. Testing was initiated one month later. Serum was tested for the presence of antibodies reactive to the transgene protein by dot blot, by Elisa, as well as neutralizing activity that can reduce viral infectivity several fold, as tested by plaque assays of the virus after it has been exposed to the serum. Injections of 10 μg are repeated monthly for several months, to boost the response, each time also testing the serum. After several months the mice are sacrificed and their spleens tested for $CD3^+$ $CD8^+$ T cells which proliferate and secret γ-interferon and induce cytotoxic in response to the activation with gD.

In all cases, the transgenes are driven by the CMV promoter and can be expressed in mouse cells. (ii) The amplicon-6 gp160-REV vectors are tested for the ability to neutralize pseudo HIV replication. (iii) The mice will be tested for the ability to mount immune activity, secretion of interferon and cytolytic activity in response to exposure to the respective antigens

The second type of vaccination is with the packaged infectious virus, which was derived ex vivo, in the presence of the helper virus (see below).

It will be readily apparent to the person skilled in the art that the amplicon vectors of the invention could be used for vaccination against many other proteins in addition to those tested. For example, similar use of the amplicon-6 vectors can be done for other herpesviruses utilizing their respective cell surface proteins (e.g., varicella zoster virus, human cytomegalovirus, Epstein Barr virus and HHV-8). It can also be done against other (non-herpes) viruses. Additionally, vaccination employing the vector can involve other diseases characterized by defined antigens, such as the muc-1 protein expressed in breast cancer and the prostate specific antigen (psa) which is highly expressed in prostate cancer. The amplicon vectors can be delivered to the T cells and dendritic cells by DNA vaccination and by infection with amplicon virions.

Example 10: Vaccination using amplicon virions

The second and third types of vaccinations will involve the introduction of cell associated and cell free amplicon-6 vectors carrying the transgene.

For experiments in monkeys, the cell-associated vector will first be derived *ex vivo*, employing monkey PBMC (peripheral blood mononuclear cells). The infected cells will be introduced into the monkey by injection, as described above. Alternatively, filtered, cell-free vectors will be introduced intravenously into Rhesus monkey macaques. The vaccination will be repeated twice to boost the immune response, The serum of vaccinated animals will be tested for production of antibodies by immunoblotting, ELISA, and virus neutralization. Furthermore, CTL assays will be done, testing chromium release.

Effector cells will be prepared by prior incubation with PBMC infected cultures carrying the transgene antigen. The effector cells will be tested for proliferation, secretion of interferons and CTL activity (by chromium release).

For injection into human subjects, packaged amplicon virions are prepared as described in Materials and Methods, and propagated in pathogen-free cells. The vaccine virus may be prepared for vaccination in various ways known in the art. For example, the virus may be suspended in a solution of serum-free,

antibiotic-free culture medium containing virus stabilizers, and then lyophilized. Alternatively, the virus may be stored at 4°C until use.

Prior to injection the lyophilized viral preparation is reconstituted in double distilled water, and injected to the subject. Each injection dose should contain between 10^4-10^8 TCID (Tissue Culture Infection Dose) of virus, preferable between 10^5-10^7 TCID. However, the specific injection doses vary according to the specific antigen to be delivered by the amplicon vector, and should be determined by routine experimentation. The helper virues (preferably HHV-6A) is co-injected. The amount of co-injected helper virus (similarly prepared) should also be determined in the same manner.

Injection is preferably intramuscular, although other modes of injection may also be used, as described above. The injection regimen may vary for each antigen, and should be assayed by routine experimentation as is known in the art. One possible injection regimen may be injection at times 0, 1, 3 and 6 months.

CLAIMS:

1. A recombinant DNA vector comprising:

(One) a DNA sequence derived from HHV-6 or HHV-7, said DNA sequence comprising an origin of replication, a promoter sequence which induces expression in a lymphocyte cell host and a cleavage and packaging signal; and

(Two) optionally a foreign nucleic acid sequence;

wherein administration of said DNA vector to a mammal results in an immune response in said mammal.

2. The vector of Claim 1, wherein said immune response is elicited against an amino acid product encoded by a DNA sequence selected from:

(One) the DNA sequence of Claim 1(a) or fragments thereof; or

(Two) the nucleic acid sequence of Claim 1(b) or fragments thereof.

3. The vector of Claim 2, wherein at least part of said expressed sequence is targeted to the cell membrane.

4. The vector of Claim 1 or 2, wherein said DNA sequence is amplicon-6.

5. The vector of Claim 1 or 2, wherein said DNA sequence is Tamplicon-7.

6. The vector of Claim 2, wherein at least part of said expressed sequence is secreted outside of the cell.

7. The vector of Claim 1 or 2, wherein said foreign nucleic acid molecule is glycoprotein D (gD).

8. The vector of Claim 1 or 2, wherein said foreign nucleic acid molecule is gp160.

9. The vector of Claim 1 or 2, wherein said foreign nucleic acid molecule is REV.

10. The vector according to any one of Claims 1 to 9, wherein said vector is capable of autonomous replication.

11. The vector according to any one of Claims 1 to 9, wherein said vector is not capable of self replication and is used in combination with a helper virus.

12. The vector according to Claim 11, wherein said helper virus is a lymphotropic virus.

13. The vector according to Claim 12, wherein said helper virus is HHV-6A.

14. The vector according to Claim 12, wherein said hevirus is HHV-7.

15. The vector according to any one of Claims 1 to 14, wherein said vector is packaged in a virion particle.

16. A method for generating an immune response in a mammal, said method comprising the steps of:

(a) providing a vector according to any one of Claims 1 to 15; and

(b) introducing said vector into the body of said mammal;

wherein said introduction results in an immune response in said mammal.

17. A vaccine comprising an effective amount of the DNA vector of any one of Claims 1 to 15.

18. A lymphotropic vector, optionally carrying a foreign DNA sequence, wherein the administration of said lymphotropic vector to a mammal results in an immune response.

19. A pharmaceutical composition comprising an effective amount of the vector of any one of claims 1 to 15, and a pharmaceutically acceptable carrier.

20. The pharmaceutical composition of Claim 19 for inducing an immune response in a mammal.

21. The pharmaceutical composition of Claim 19 for inducing an immune response agains a nucleic acid sequence of interest in a mammal.

22. The pharmaceutical composition of Claim 19, further comprising a helper virus.

23. The pharmaceutical composition of Claim 22, wherein said helper virus is HHV-6A.

24. The pharmaceutical composition of Claim 22, wherein said helper virus is HHV-7.

25. A method of treating a disease in a subject in need comprising administering the pharmaceutical composition of any one of Claims 19 to 24.

26. A kit comprising the pharmaceutical composition of any one of Claims19 to 24, and instructions for use thereof.

Figure 1
AMPLICON-TYPE VECTORS

amplicon-6 **(HHV-6)**
Tamplicon-7 **(HHV-7)**

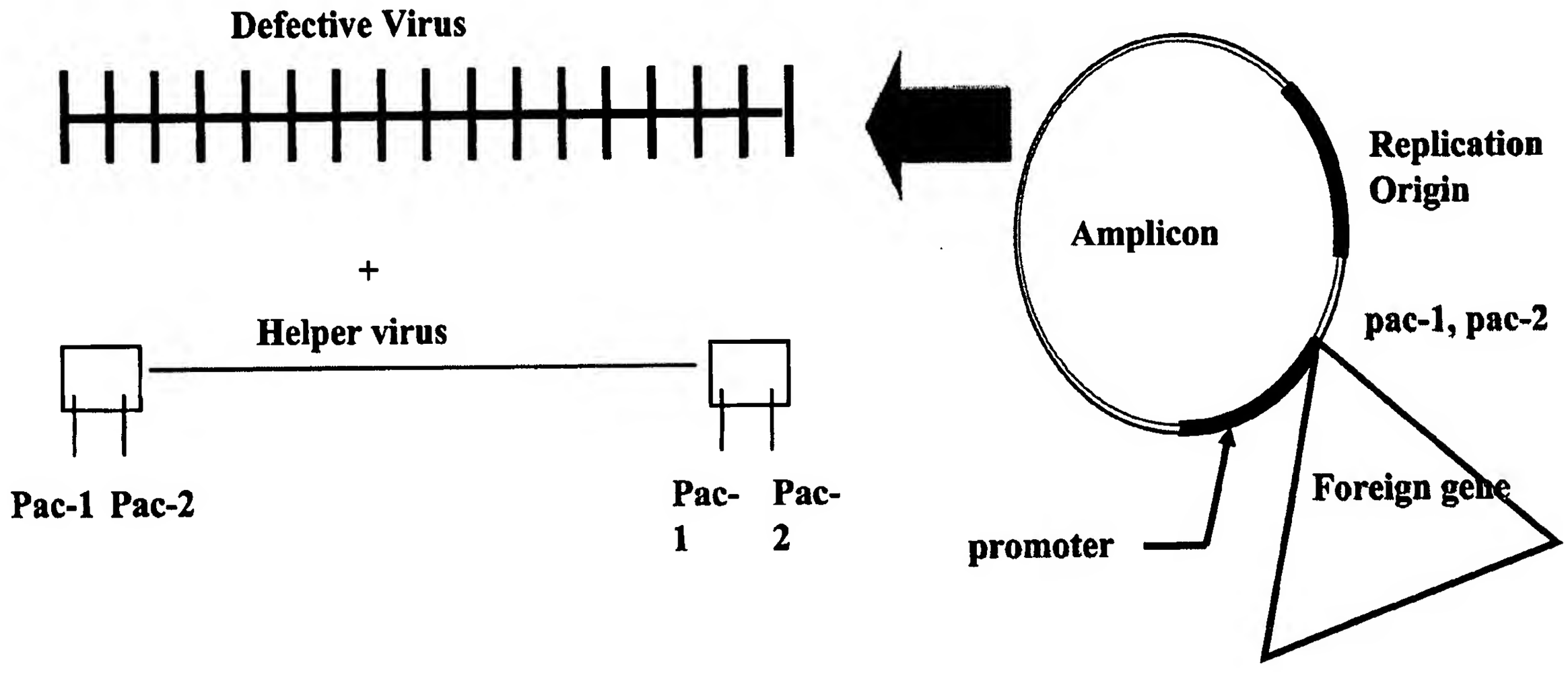
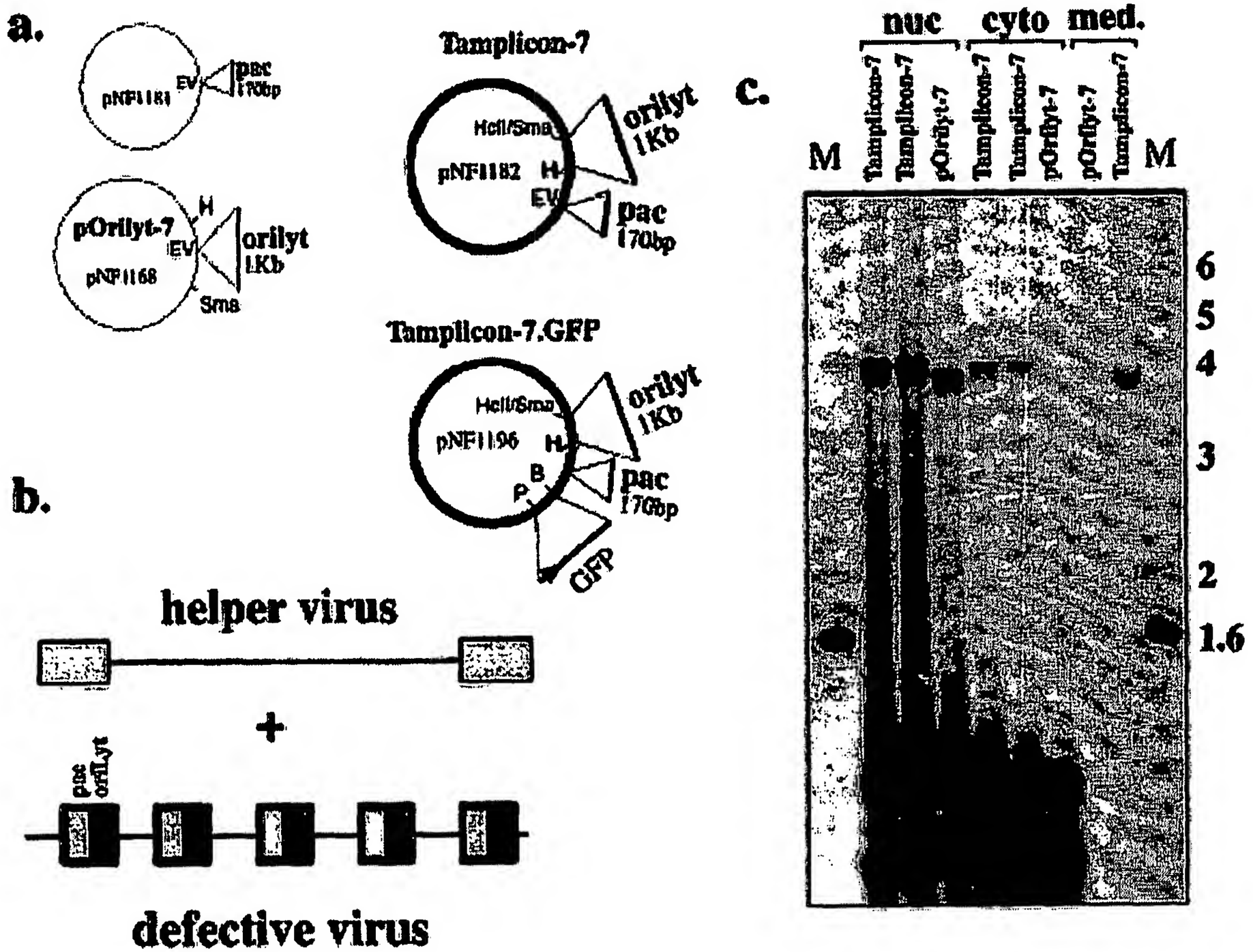


Figure 2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Sma
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pNF1182
H
EV
orilyt
1Kb
pac
170bp
Tamplicon-7.GFP
HcII/Sma
pNF1196
H
B
orilyt
1Kb
pac
170bp
GFP
b.
helper virus
+
pac
orilyt
defective virus
c.
nuc
cyto
med.
M
Tamplicon-7
Tamplicon-7
pOrilyt-7
Tamplicon-7
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Tamplicon-7
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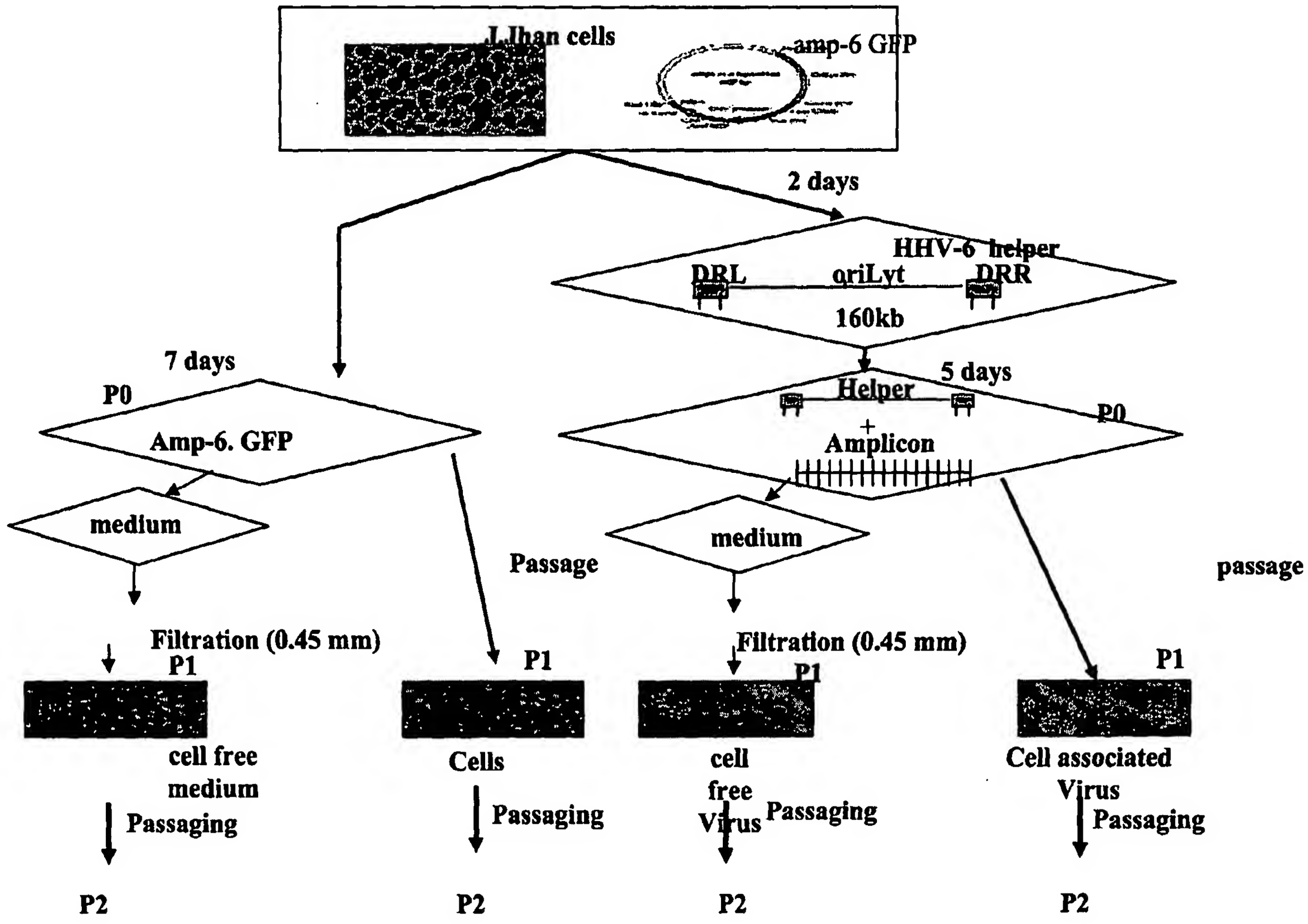
6
5
4
3
2
1.6

Figure 3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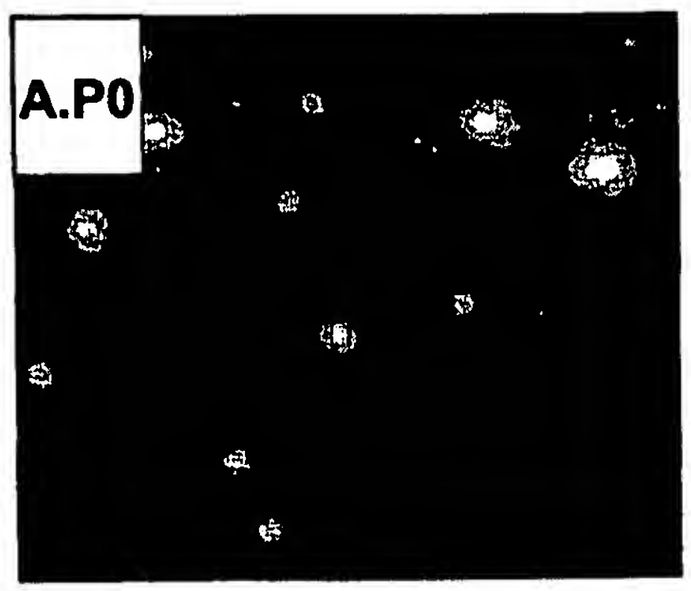
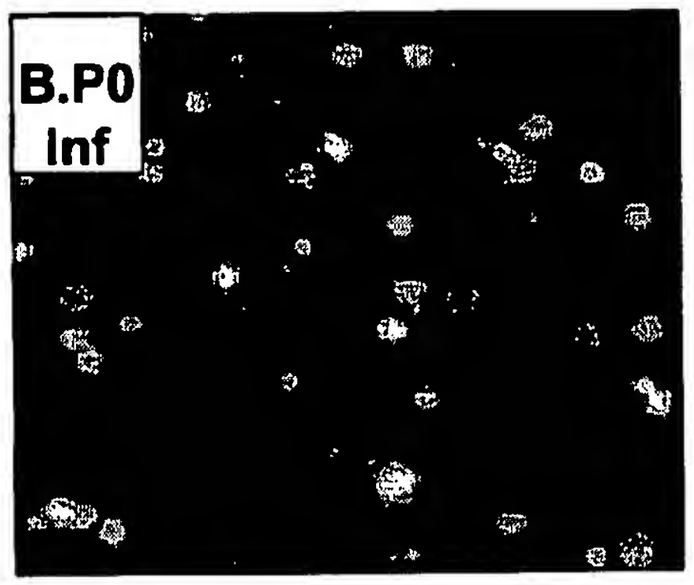
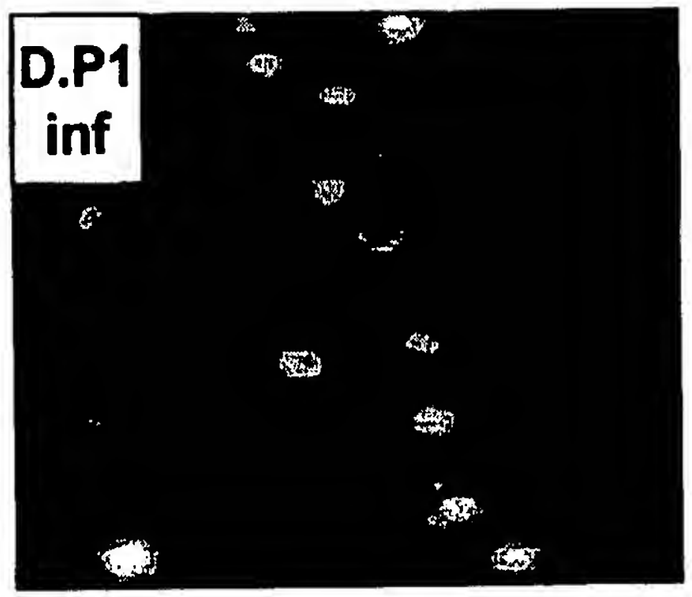
Helper
+
Amplicon
P0
medium
medium
Passage
passage
Filtration (0.45 mm)
Filtration (0.45 mm)
P1
P1
P1
P1
cell free
medium
Cells
cell
free
Virus
Cell associated
Virus
Passaging
Passaging
Passaging
Passaging
P2
P2
P2
P2

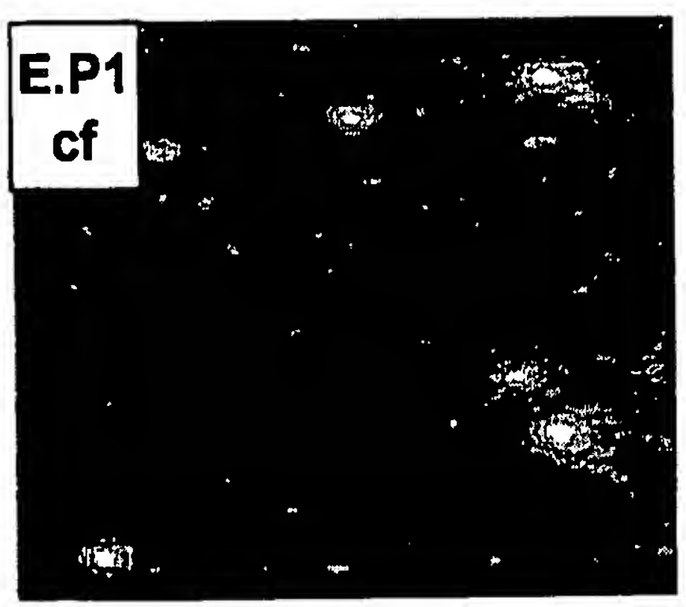
Figure 4


A.P0


B.P0
Inf


C.P1


D.P1
inf


E.P1
cf

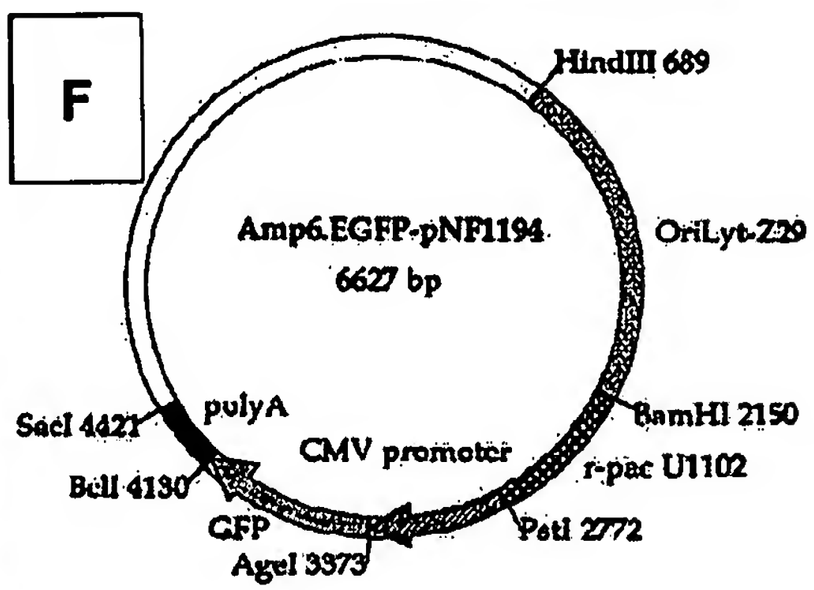
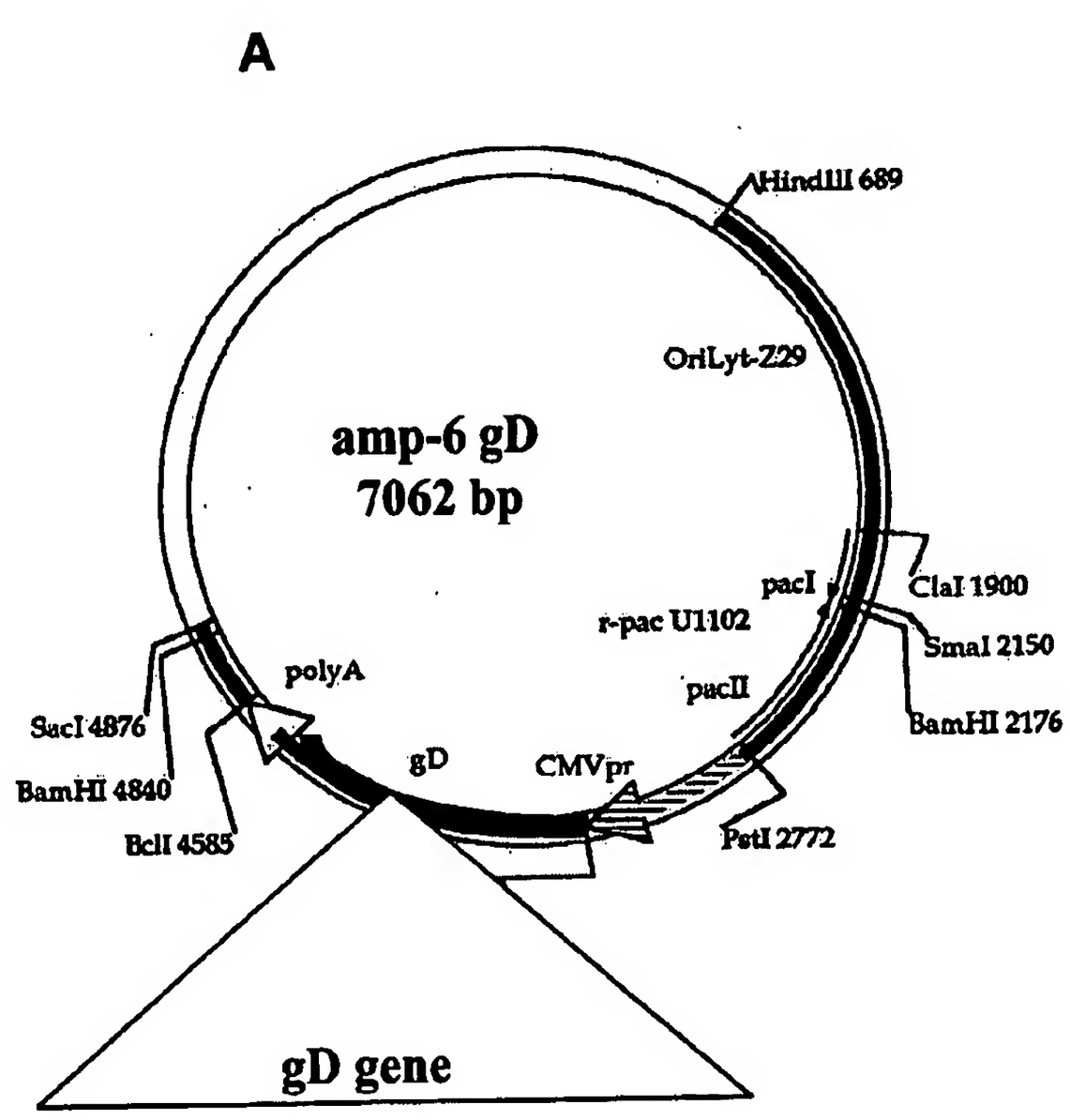
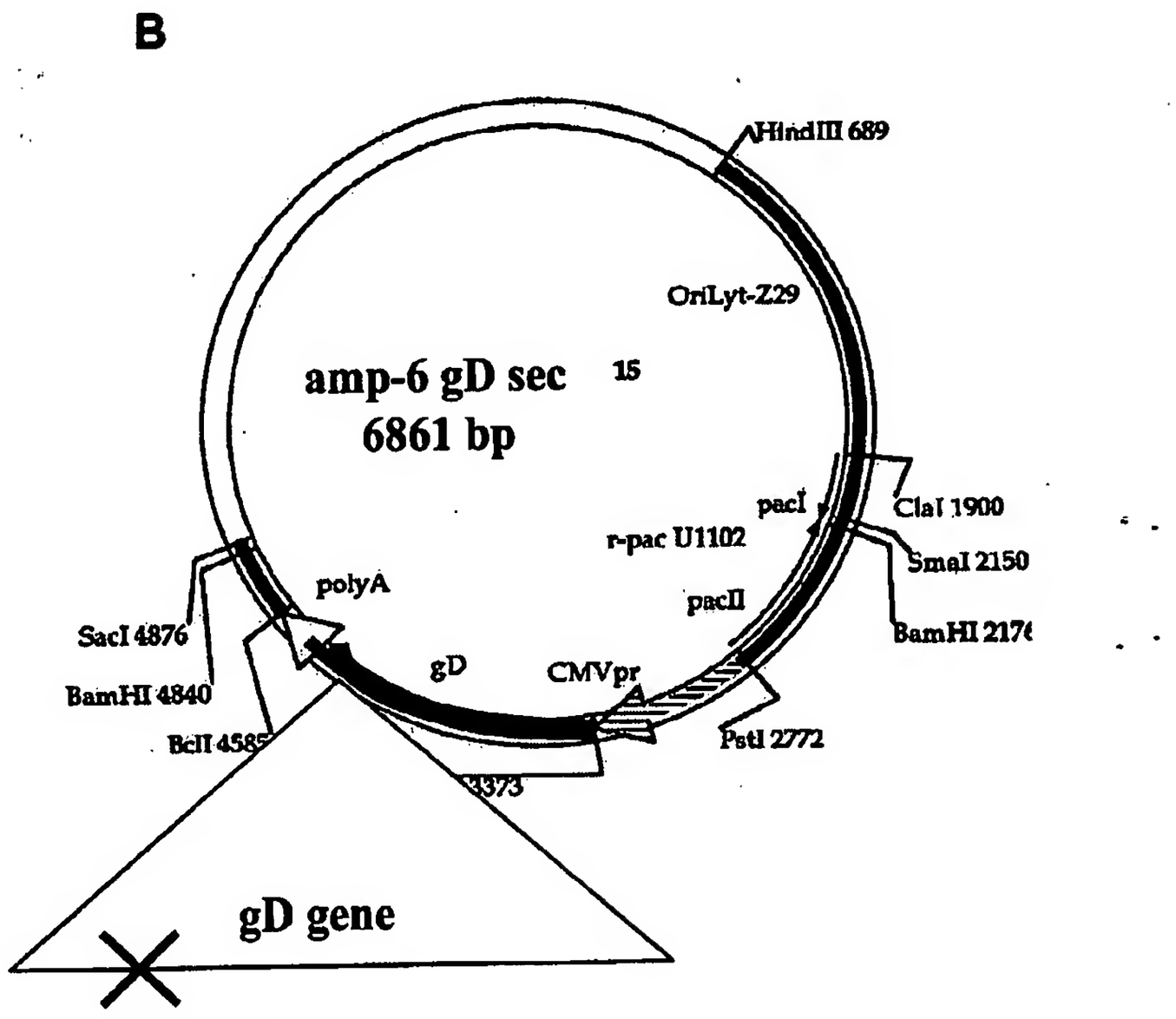

F
HindIII 689
Amp6.EGFP-pNF1194
6627 bp
OriLyt-Z29
BamHI 2150
r-pac U1102
PstI 2772
AgeI 3373
GFP
BclI 4130
CMV promoter
polyA
SacI 4421

Figure 5





201bp Deletion of Trans Membrane region

Figure 6

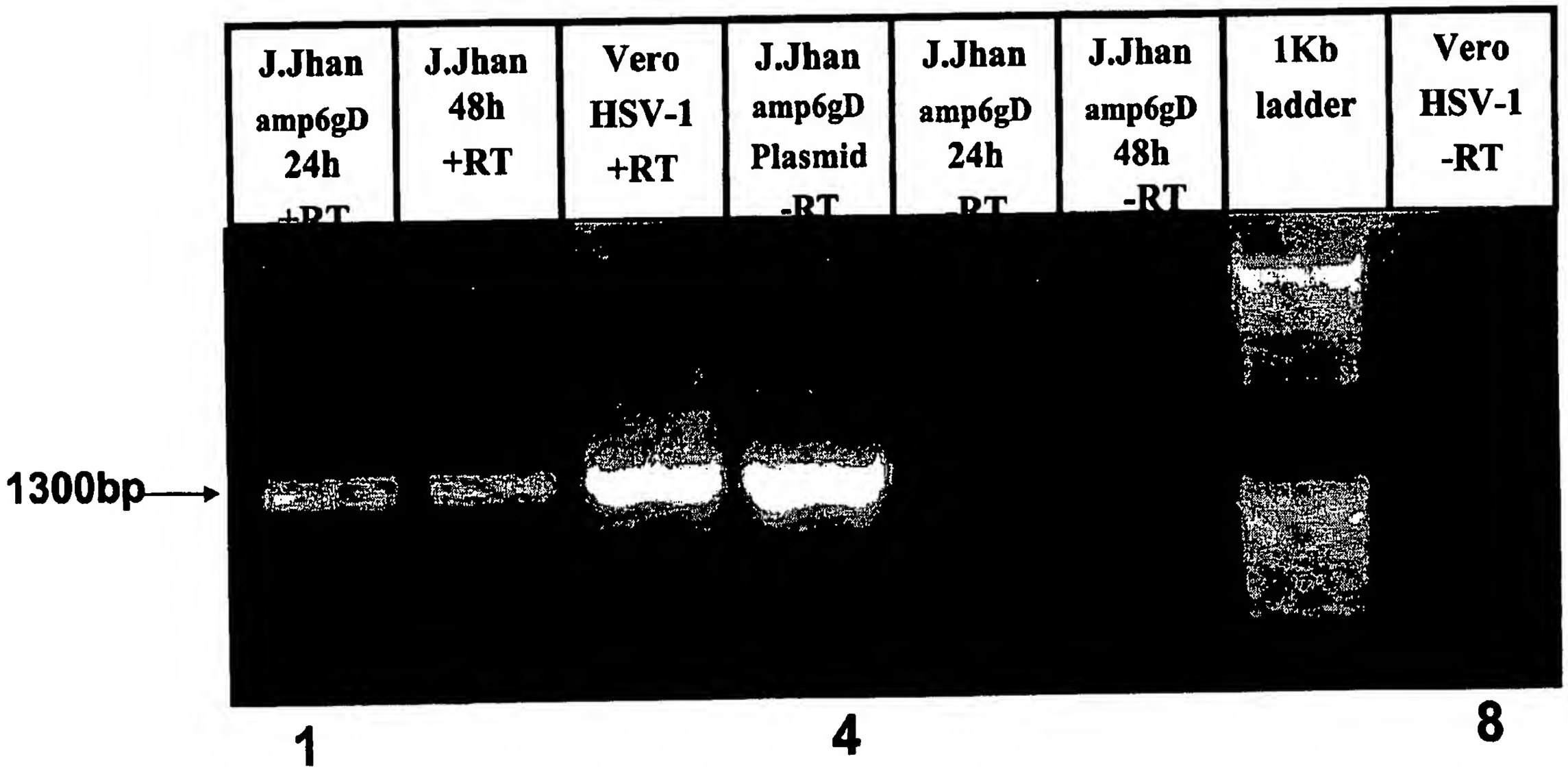

J.Jhan amp6gD 24h +RT
J.Jhan 48h +RT
Vero HSV-1 +RT
J.Jhan amp6gD Plasmid -RT
J.Jhan amp6gD 24h -RT
J.Jhan amp6gD 48h -RT
1Kb ladder
Vero HSV-1 -RT
1300bp
1
4
8

Figure 7

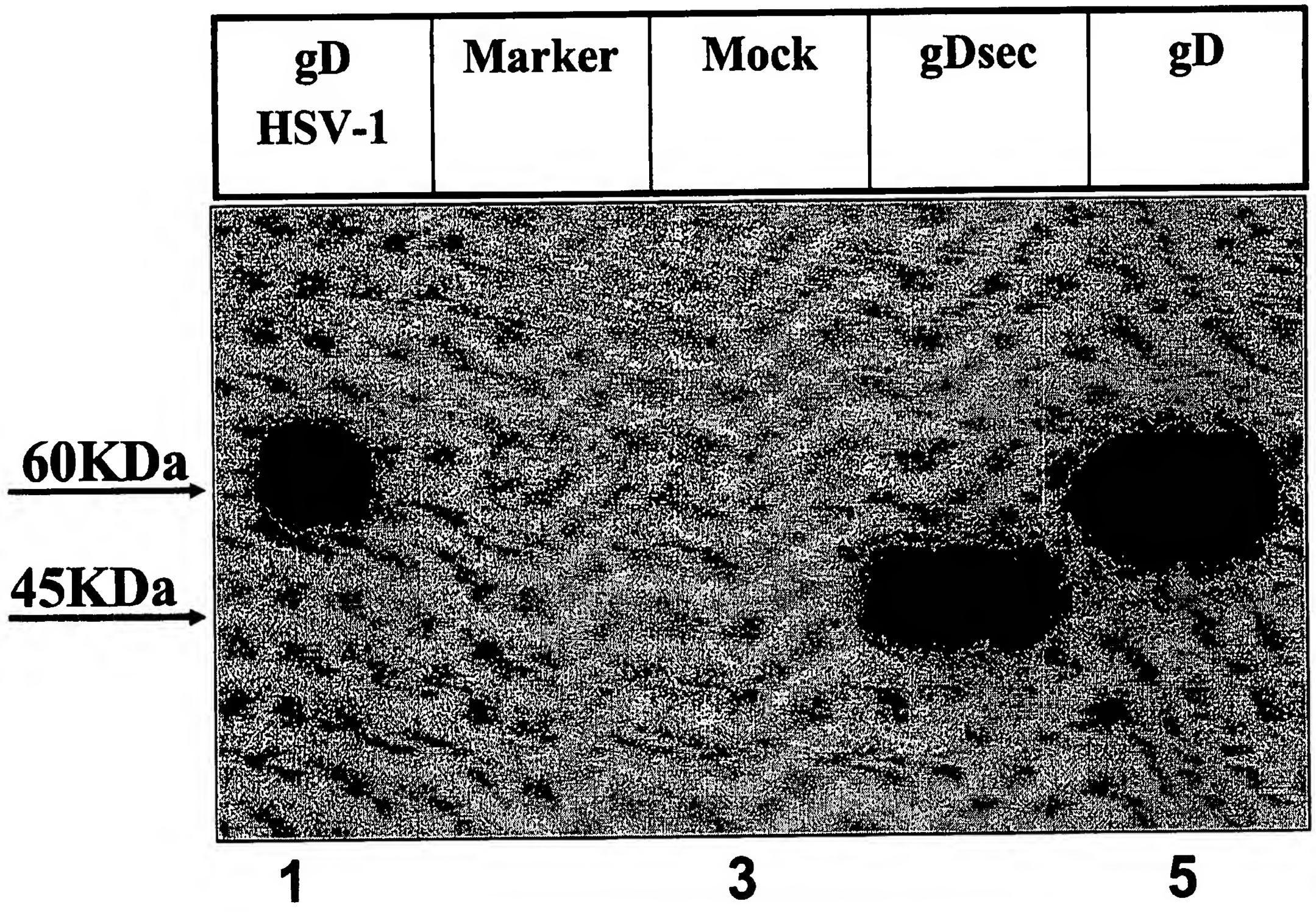


Figure 8

				Passage - 0		Passage - 1			
	gD HSV-1	**Marker**	**HHV-6A**	**gD**	**gD**	**gD Medium**	**gD Medium**	**gD**	**gD**
Amp-6	-	-	-	+	+	+	+	+	+
HHV-6A	-	-	+	-	+	-	+	-	+

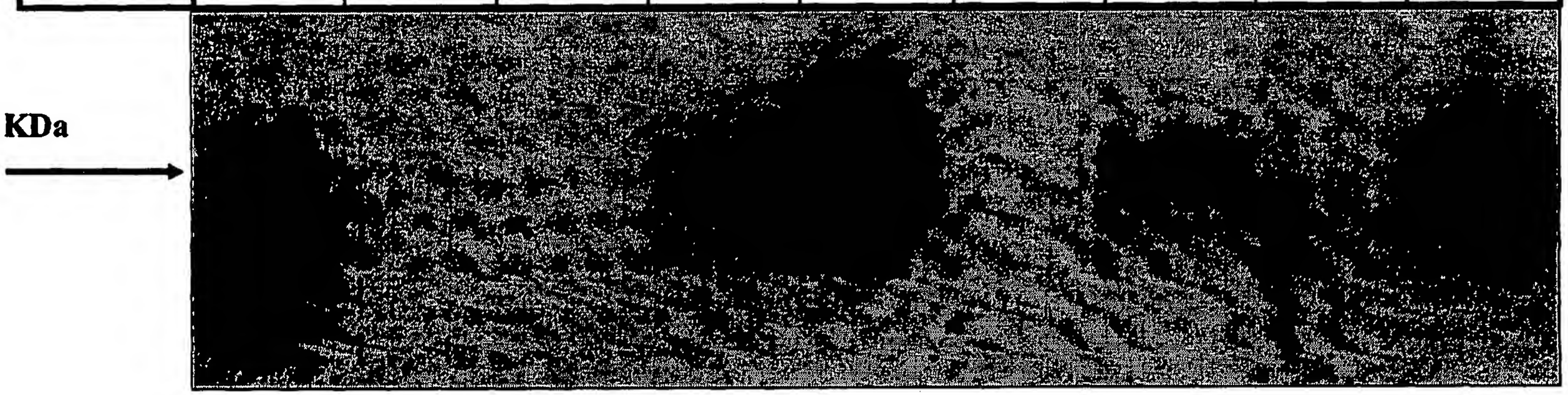


Figure 9

			Passage - 0		Passage - 1			Passage - 2
	gD HSV-1	Marker	gDsec	gDsec	gDsec Medium	gDsec	gDsec	gDsec
Amp-6	-	-	+	+	+	+	+	+
HHV-6A	-	-	-	+	+	-	+	+

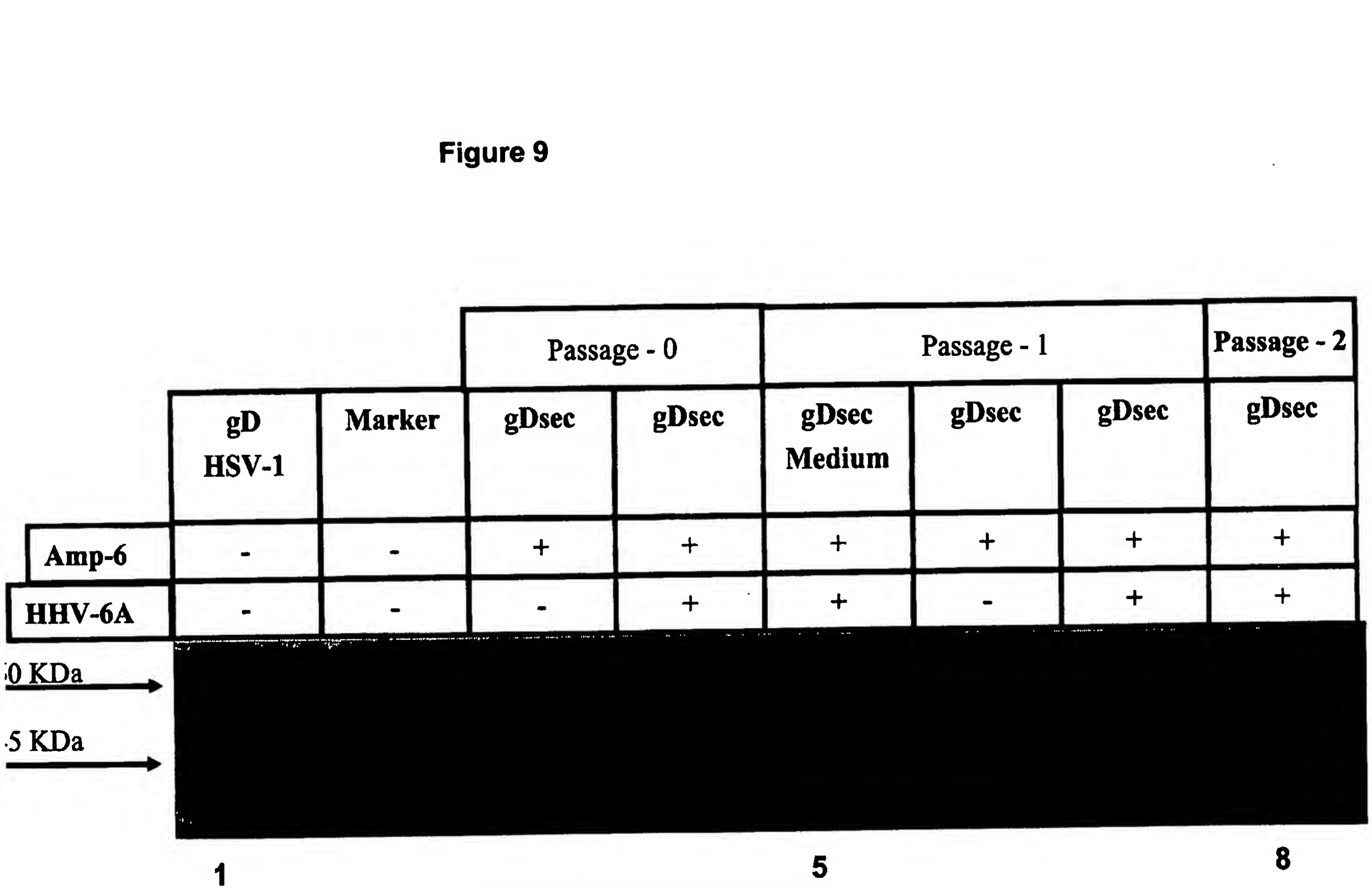


Figure 10

	gD HSV -1	Marker	-gDs P-0 48h	-gDsec TCA 48h	-gDsec TCA 7 days	gDsec TCA	-gD P-0 48h	-gD TCA 48h	-gD TCA 7 days	-gD TCA
Amp-6	-	-	+	+	+	+	+	+	+	+
HHV-6A	-	-	-	-	-	+	-	-	-	+

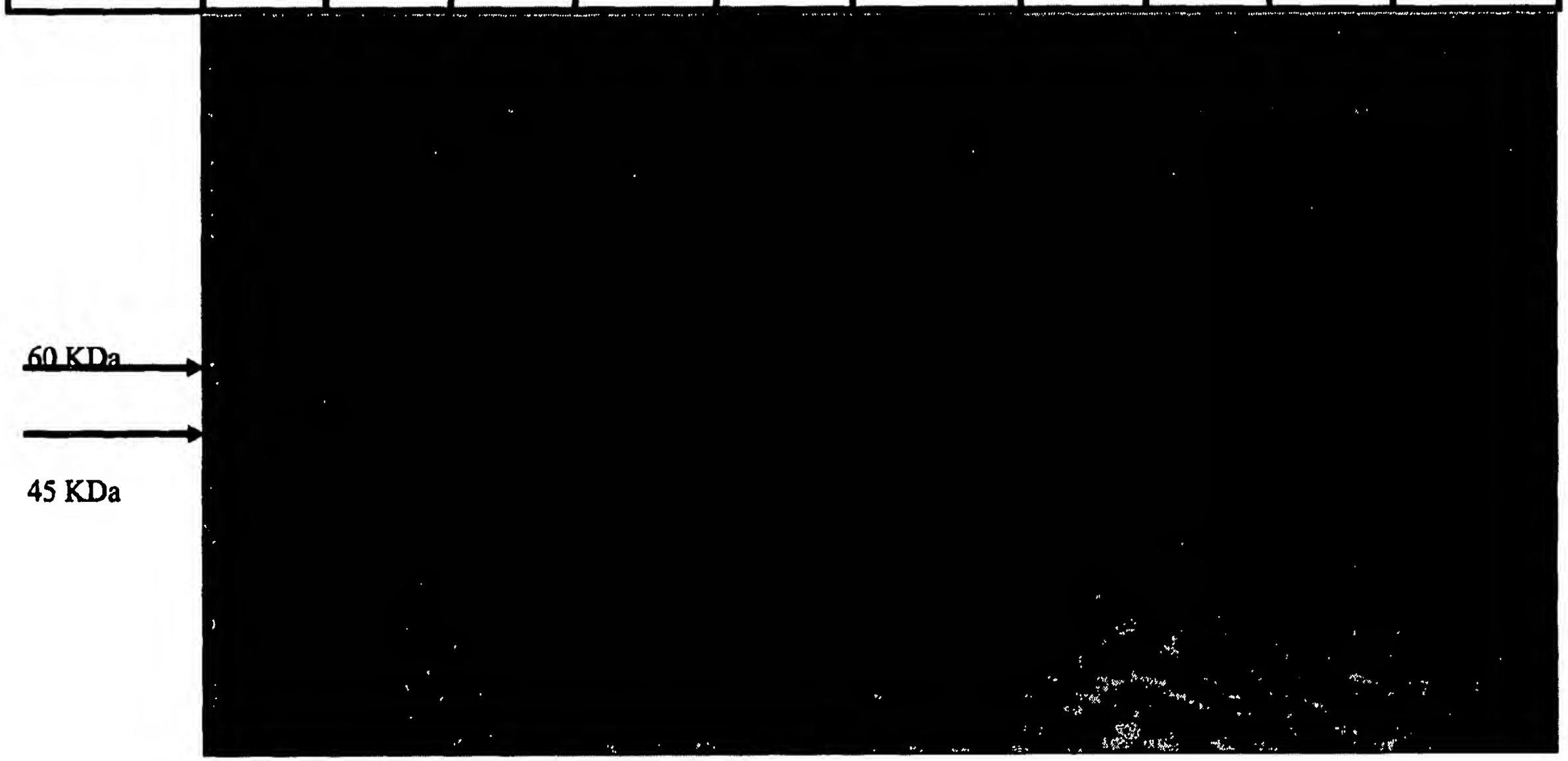


Figure 11

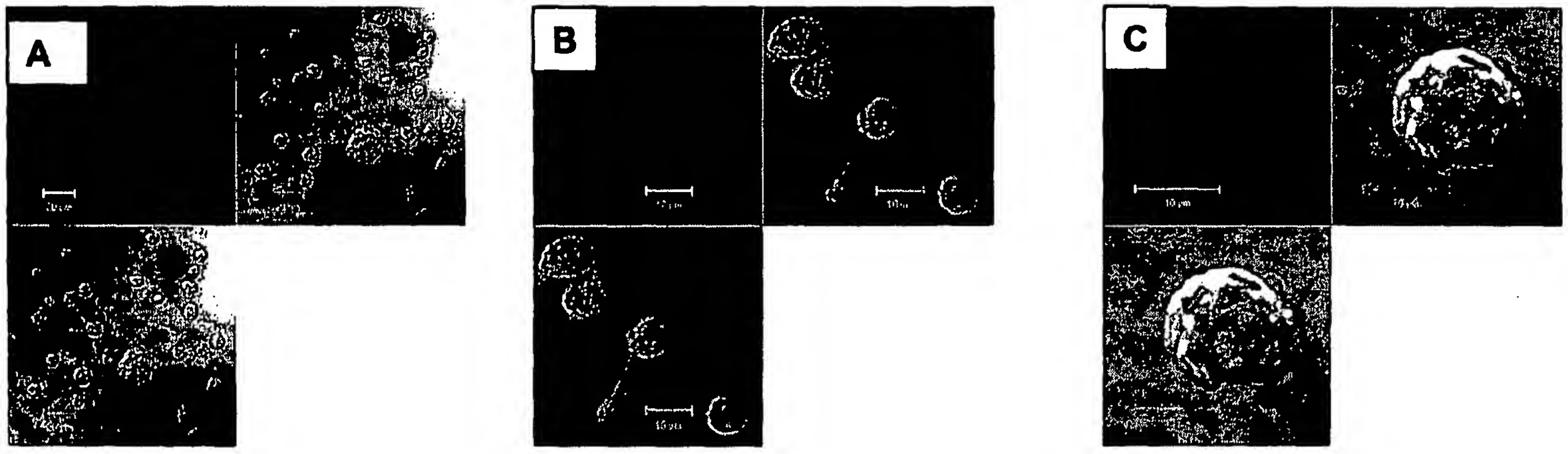


Figure 12

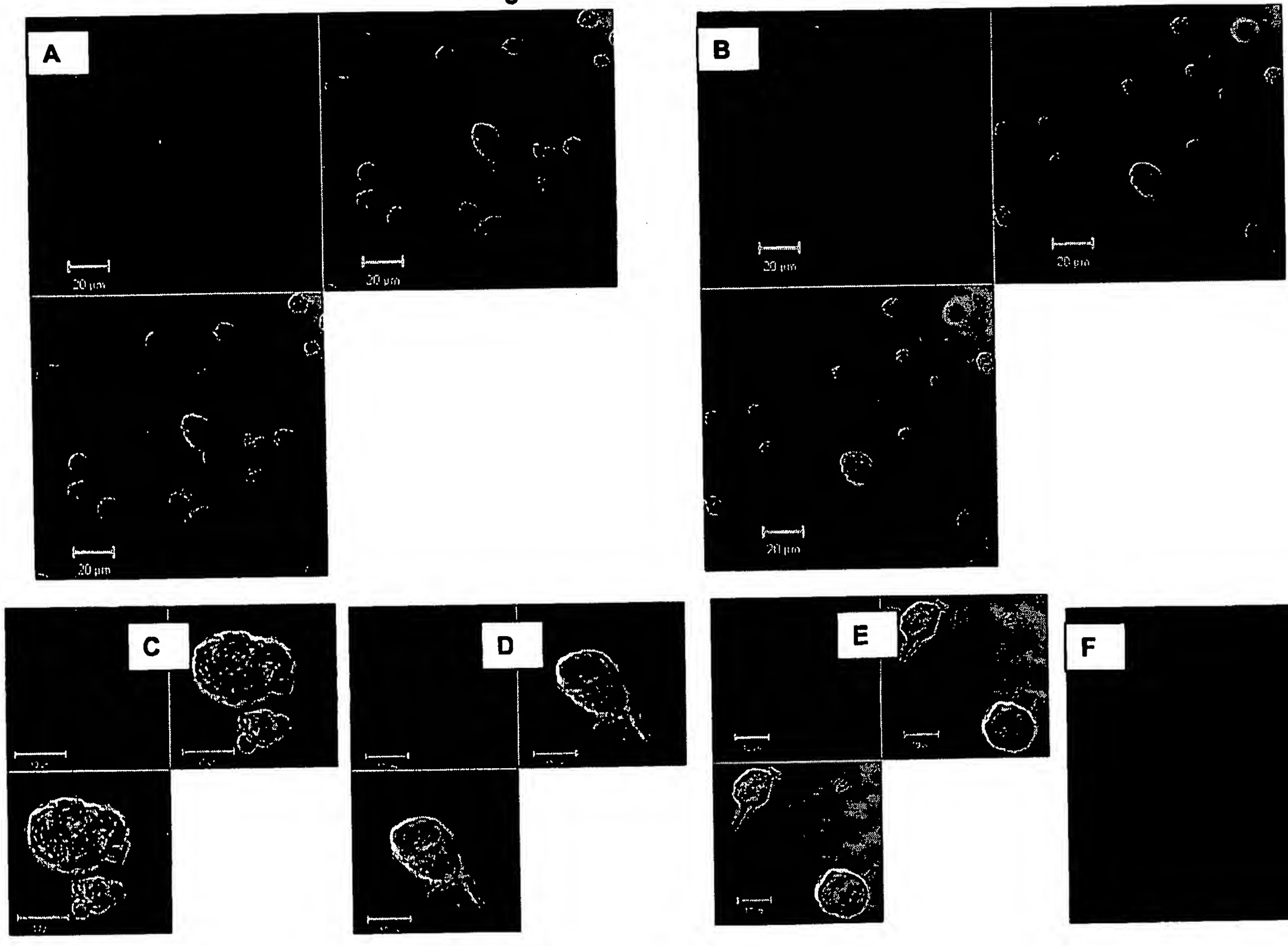


Figure 13

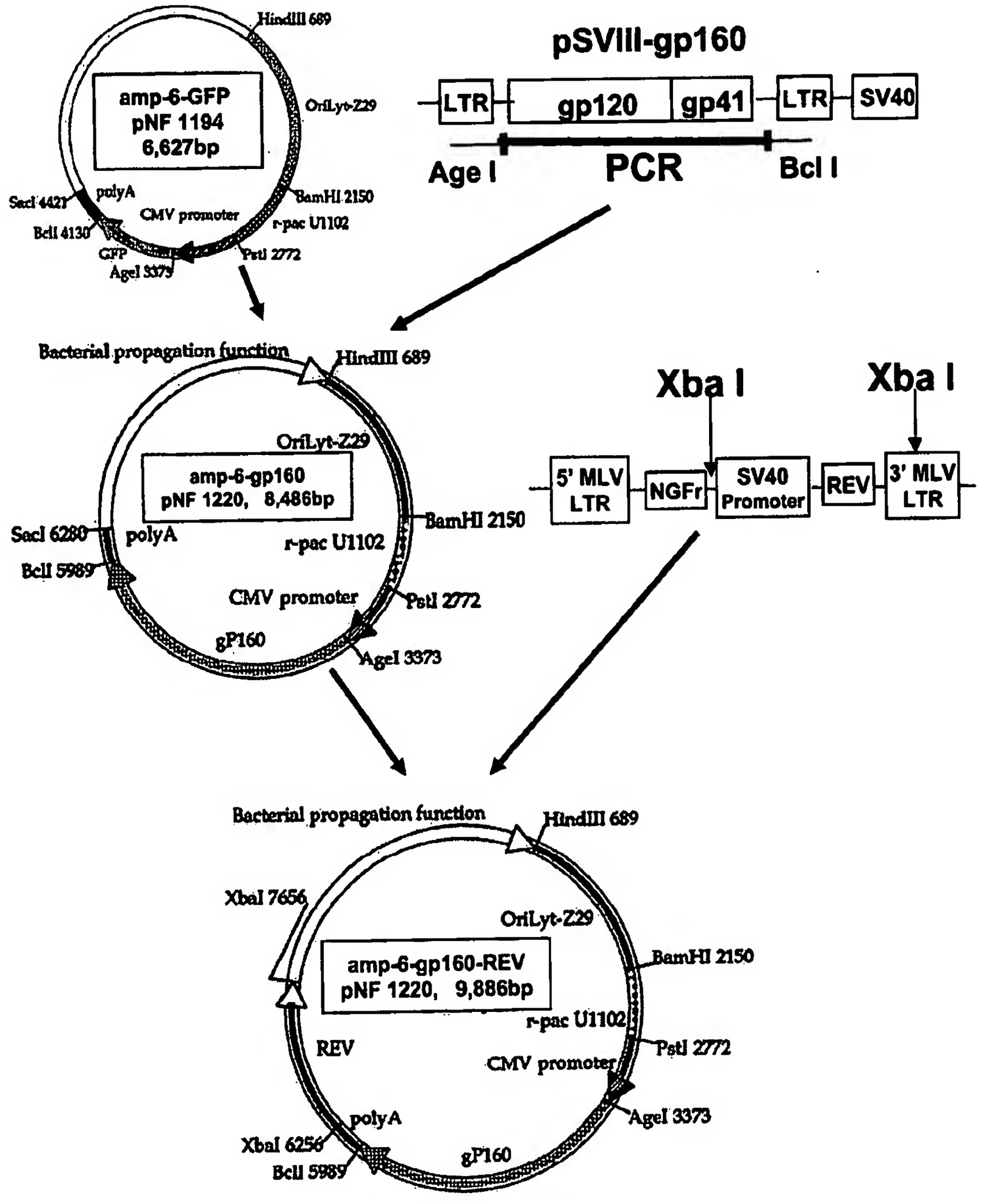

amp-6-GFP
pNF 1194
6,627bp
HindIII 689
OriLyt-Z29
BamHI 2150
r-pac U1102
PstI 2772
AgeI 3373
GFP
CMV promoter
polyA
SacI 4421
BclI 4130
pSVIII-gp160
LTR
gp120
gp41
LTR
SV40
Age I
PCR
Bcl I
Bacterial propagation function
HindIII 689
OriLyt-Z29
amp-6-gp160
pNF 1220, 8,486bp
BamHI 2150
SacI 6280
polyA
r-pac U1102
BclI 5989
CMV promoter
PstI 2772
gP160
AgeI 3373
Xba I
Xba I
5' MLV LTR
NGFr
SV40 Promoter
REV
3' MLV LTR
Bacterial propagation function
HindIII 689
XbaI 7656
OriLyt-Z29
amp-6-gp160-REV
pNF 1220, 9,886bp
BamHI 2150
r-pac U1102
REV
CMV promoter
PstI 2772
AgeI 3373
polyA
XbaI 6256
BclI 5989
gP160

Figure 14

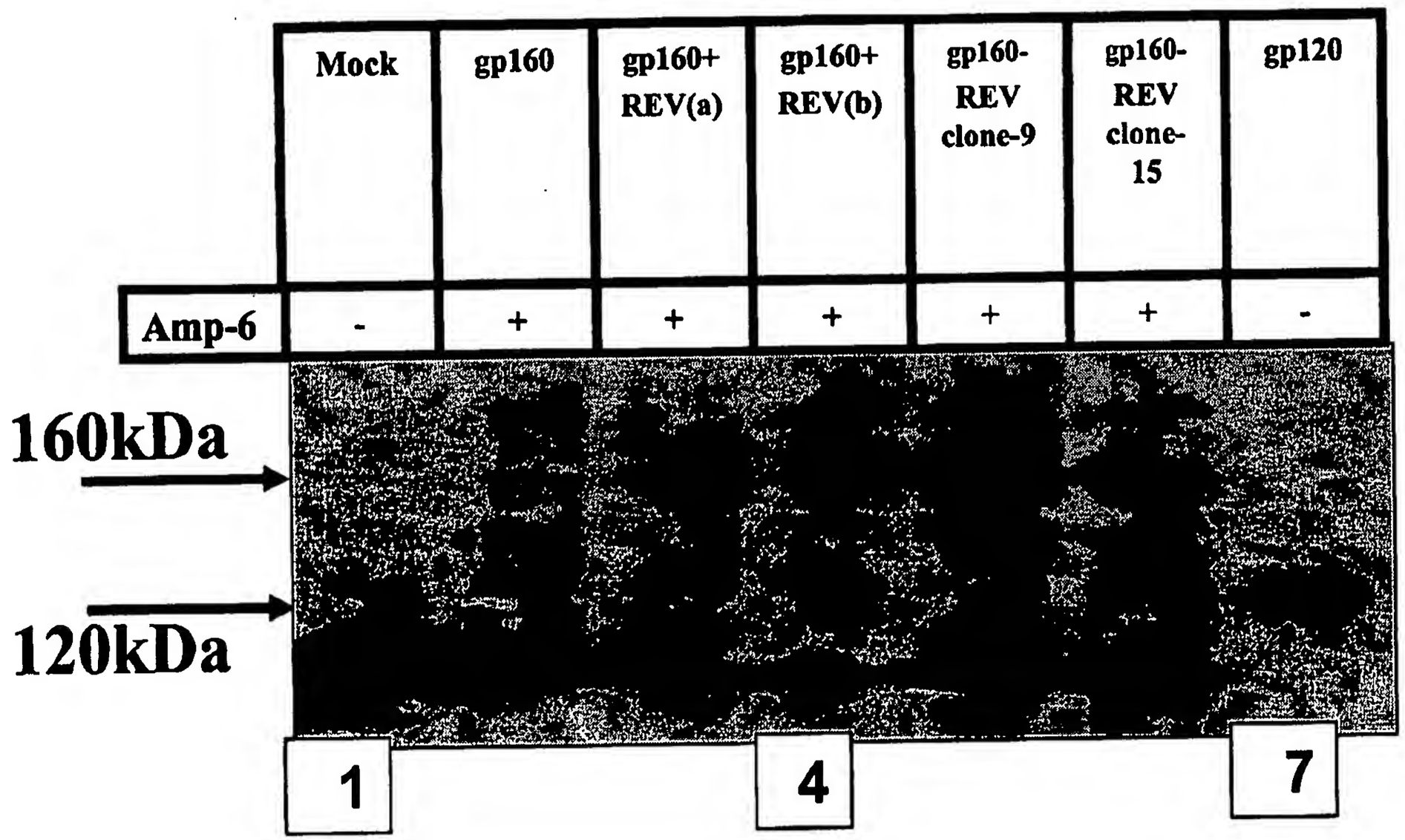

Mock
gp160
gp160+ REV(a)
gp160+ REV(b)
gp160- REV clone-9
gp160- REV clone-15
gp120
Amp-6
- + + + + + -
160kDa
120kDa
1
4
7

Figure 15

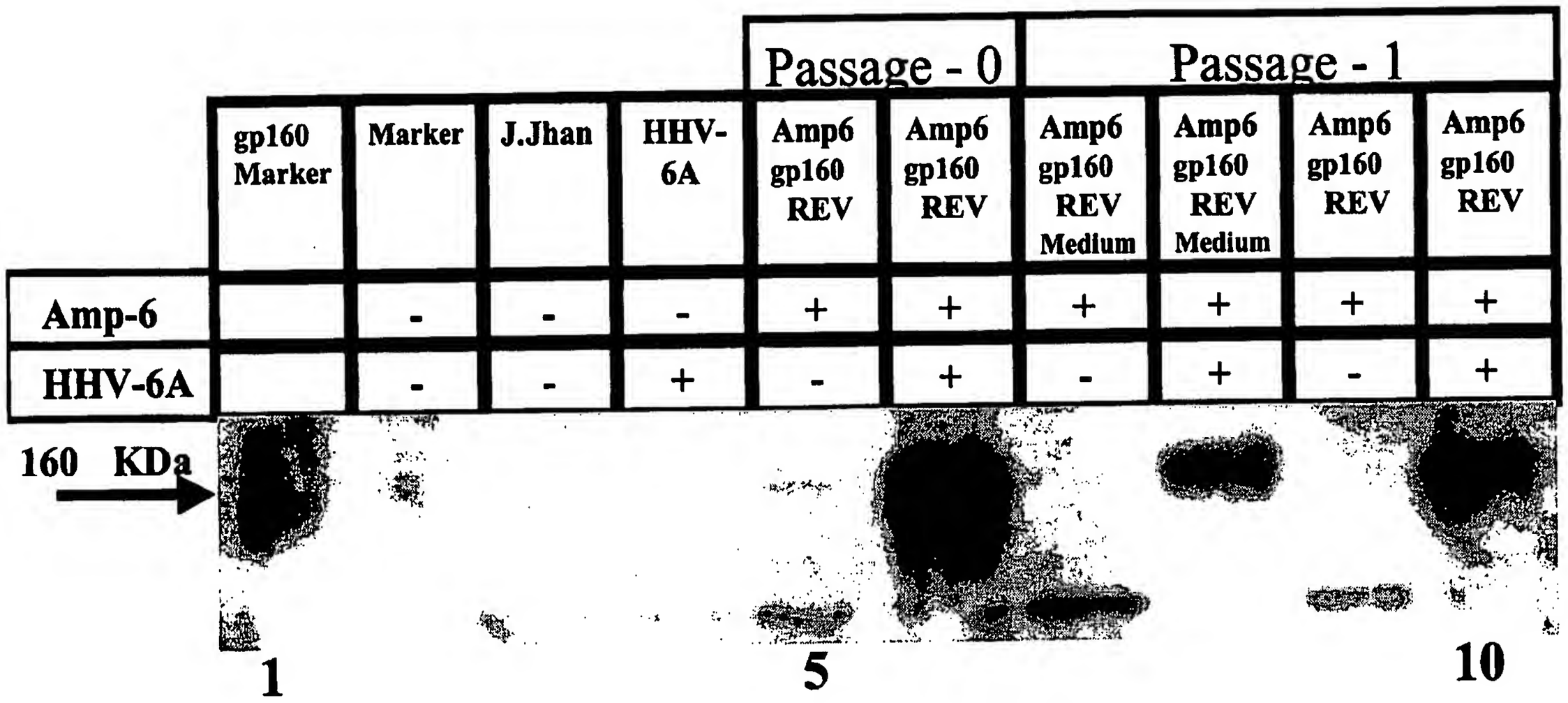

Passage - 0
Passage - 1
gp160 Marker
Marker
J.Jhan
HHV-6A
Amp6 gp160 REV
Amp6 gp160 REV
Amp6 gp160 REV Medium
Amp6 gp160 REV Medium
Amp6 gp160 REV
Amp6 gp160 REV
Amp-6
- - - + + + + + +
HHV-6A
- - + - + - + - +
160 KDa
1
5
10

Figure 16

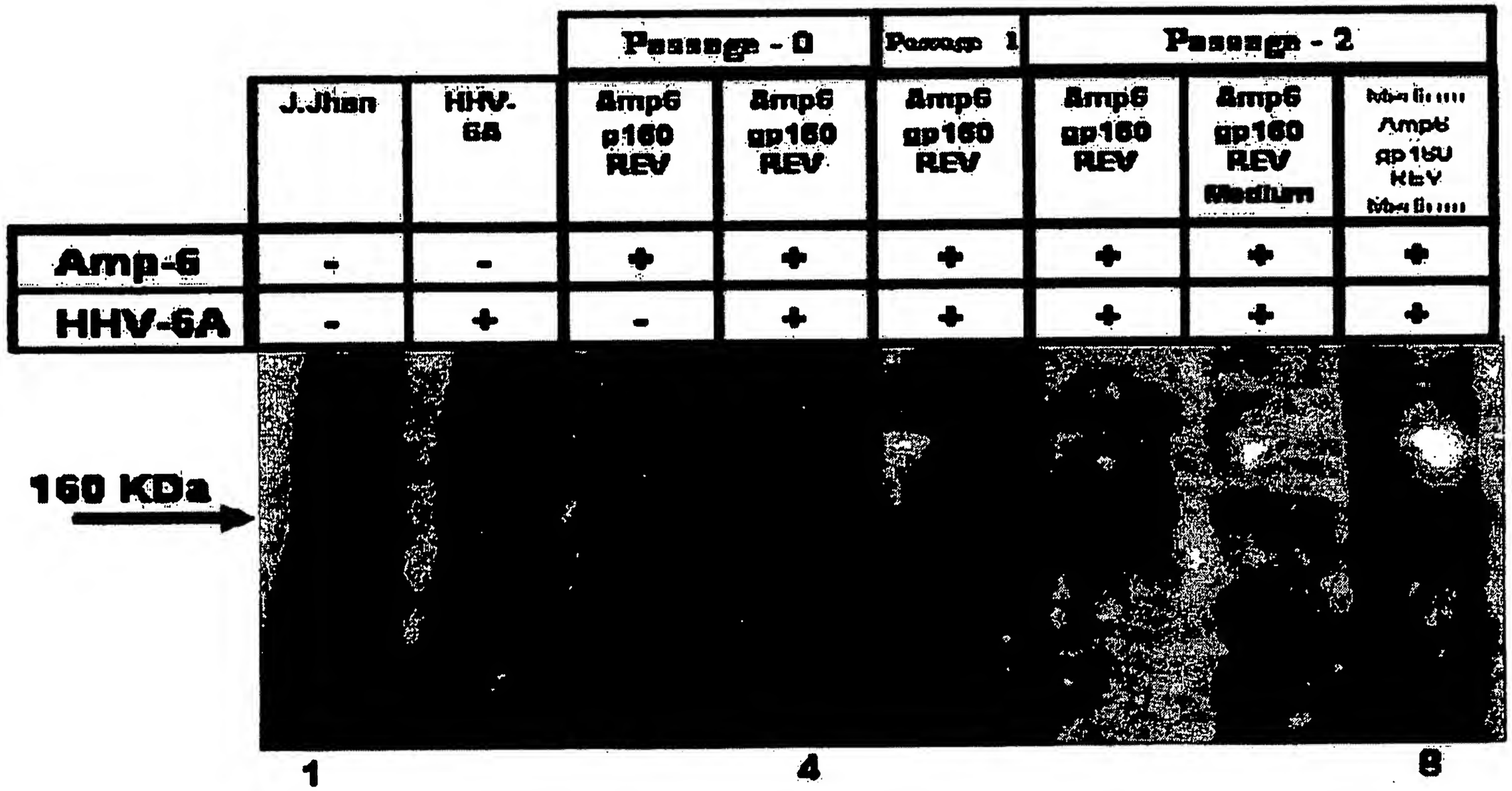

Passage - 0
Passage 1
Passage - 2
J.Jhan
HHV-6A
Amp6 gp160 REV
Amp6 gp160 REV
Amp6 gp160 REV
Amp6 gp160 REV
Amp6 gp160 REV Medium
Amp6 gp160 REV
Amp-6
HHV-6A
160 KDa
1
4
8

Figure 17

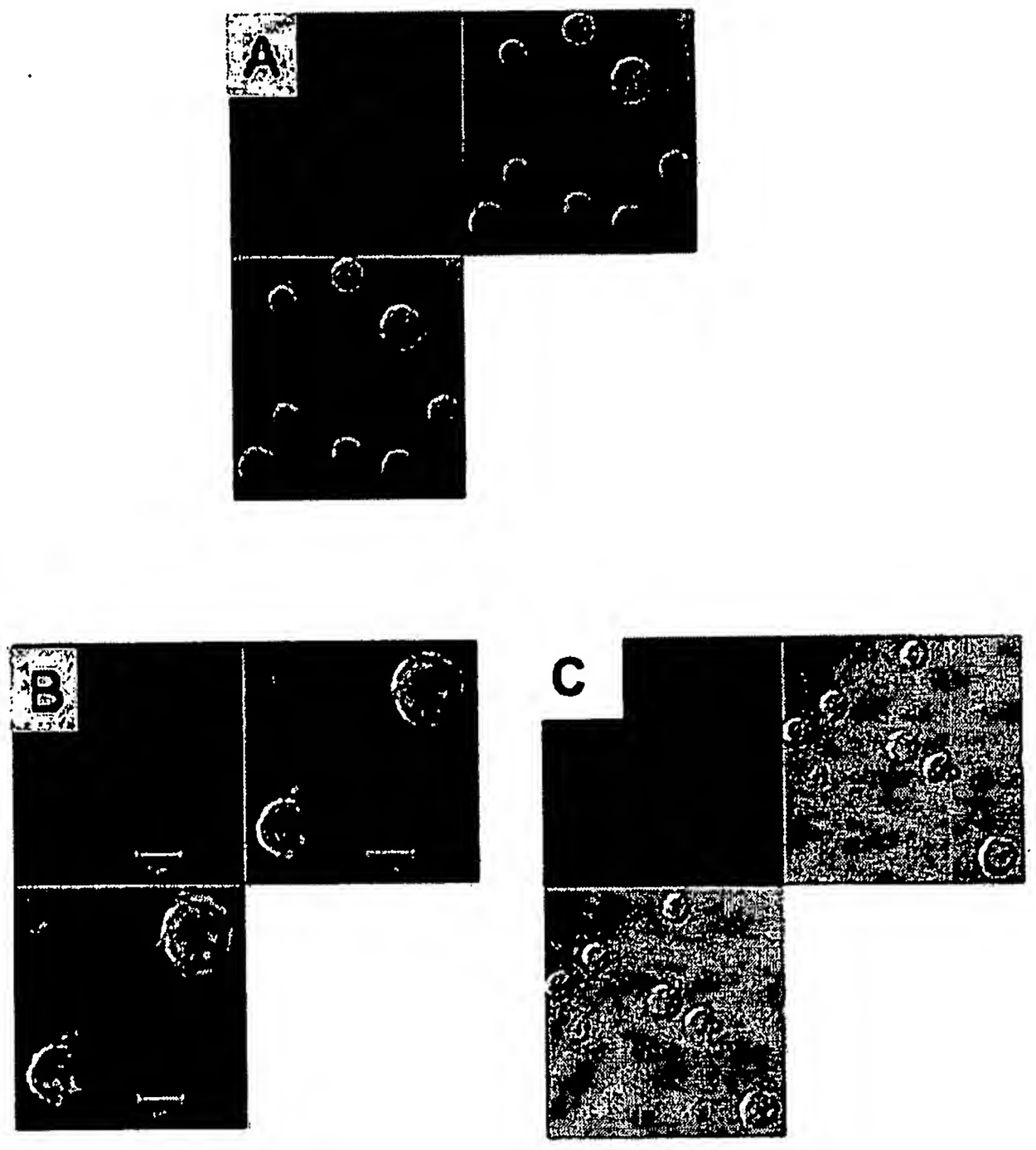
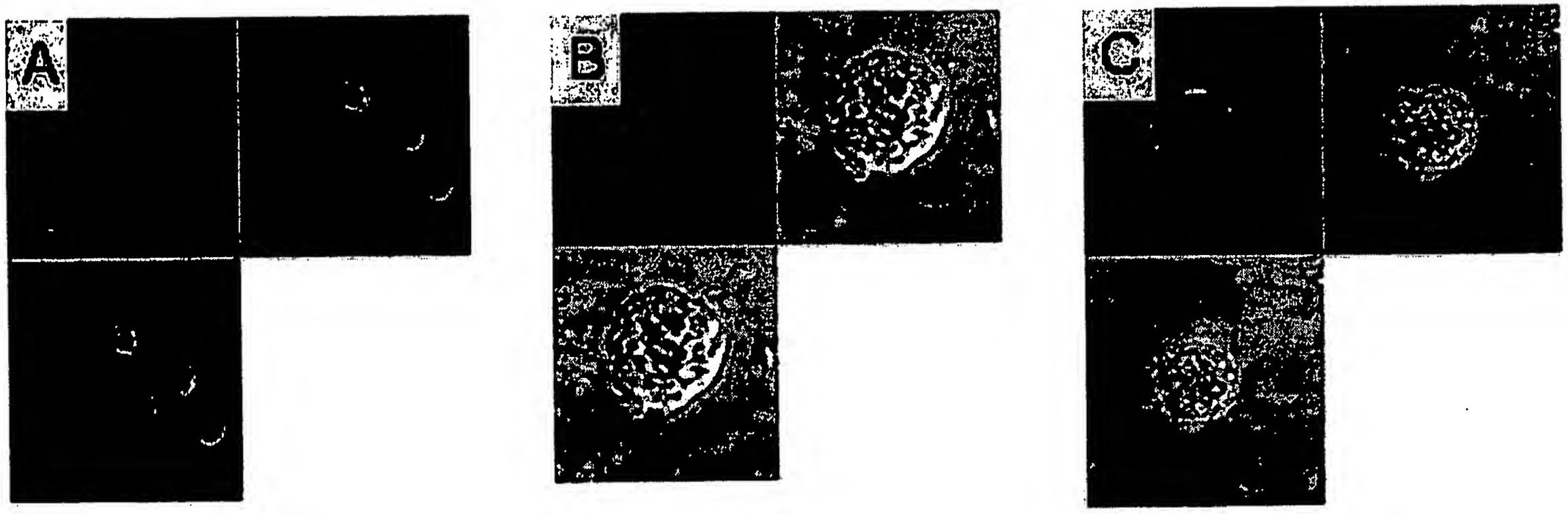


Figure 18



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